4

Results and Discussion

The results of the present investigation entitled **"Development and Evaluation of *Ulva Lactuca* based Probiotic Beverage and *in vitro* Bioavailability of Iron using Caco-2 Cell Model"** are presented and discussed under the following headings:

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PHASE I

4.1 Morphology, Nutrient and Heavy Metal Profile of the Selected Underexploited Seaweeds.

4.1.1 Morphological Characteristics and taxonomy of Underexploited Seaweeds from the Geographical Location.

The morphological characteristics and taxonomy of the four edible underexploited seaweeds *Ulva lactuca*, *Ulva reticulata*, *Gracilaria edulis,* and *Sargassum polycystum* are classified according to Fritsch *et al.,* 1935. A study of the consumption pattern of the seaweeds was conducted by Abirami *et al.,* (2012) based on which the under-exploited edible seaweeds were chosen for the study. Plate 4.1. shows the four types of seaweeds chosen for the study, and the characteristics are presented in the table.

Plate. 4.1. Underexploited Edible Seaweeds Selected for the Study.

Table 4.1: Taxonomy and Morphological Characteristics of Selected Seaweeds.

Taxonomy of the selected species	**Morphological Characteristics**				
	Type	**Colour**	**Texture**	**Shape**	**Habitat**
Ulva lactuca **Order – Ulotrichales** **Family – Ulvaceae** **Class – Chlorophyta**	Green Chlorophyta	Bright green	Silky	Circular with waved margin; has few holes in its blades; called ―Sea lettuce‖	Free-floating mass
Ulva reticulata **Order – Ulotrichales** **Family – Ulvaceae** **Class – Chlorophyta**	Green Chlorophyta	Grass green	Silky	Thin spring-like filaments; called ―Ribbon Sea lettuce‖ .	Free-floating mass
Gracilaria edulis **Order – Gracilariales** **Family – Gracilariaceae** **Class – Rhodophyta**	Red Rhodophyta	Dark purplish red	Thick and fleshy thallus, glossy and smooth	Cylindrical, blade-like, and irregularly branched; called ―Ceylon moss‖ .	Free-floating mass
Sargassum polycystum **Order – Fucales** **Family – Sargassaceae** **Class – Phaeophyceae**	Brown phaeophyceae	Light to dark brown	Slightly coarse and sticky	Short and cylindrical stem with Y-shaped proliferations	Free-floating mass

Ulva lactuca, algae are bright green in colour and silky to the touch. Although the shape of *Ulva lactuca* is quite variable, it is mostly circular or oval with waved margins and has few holes in its blade. This species of green algae is found as a free-floating mass and is commonly called ―Sea Lettuce‖ or ―green laver‖. Morphologically, it has colour variations from green to dark green depending on its occurrence i.e., underwater or at the beach. The size of the plant varies according to the maturity of the plant. The seaweed is widely found in Kanyakumari, Chinna Muttam, Vattakottai, Kootapuzhi, Perumanal, Idinthakarai, Pudumadam and Pamban, and along the entire west coast of India. The growth of plants started early in the monsoon season, and plants were found until the middle of winter (July– December) (Pereira *et al.,* 2010; Braune and Guiry *et al*., 2011).

Ulva. reticulata is an established tropical species that thrives well in clear, shallow waters. is distinctive in appearance with tubular thalli. The plant is attached to a substratum throughout its life by a hold-fast disc. It requires a warm water temperature of between 25 and 30°C, with growth occurring at a faster rate when inorganic nutrients, especially ammonia and phosphorous, are high due to its efficient nutrient uptake ability (Sharma *et al.,* 2019). *U. reticulata* is conspicuously present during peak growth in protected marine habitats, estuaries, bays, and lagoons, where salinity is around 34-35 ppt (weight of salt in water) (Déléris *et al.,* 2016). Generally, the geographical distribution of *U. reticulata* is influenced mainly by water temperature and is abundantly found over the west coast of India, across the Sri Lankan coast, and the Andaman and Nicobar Islands (Radulovich *et al.,* 2015).

G. edulis is a species of red algae that is characterized by a thick, fleshy thallus which can be either erect or prostrate. It is often a dark purplish-red colour and can appear glossy or smooth. The thallus is made up of small branches that are tightly packed together and can be either smooth or hairy in texture. It is found in many coastal regions of India and is one of the most common species of red algae on the Indian coast reported from the Islands of Andaman and Nicobar as well as Lakshadweep (Silva *et al.,* 1996; Panja *et al.* 2022), Karnataka (Gupta and Das *et al.,* 2019), Tamil Nadu (Ganesan *et al.,* 2011) and other coastal regions as well. (Lyra *et al.,* 2015; Ng *et al.,* 2017).

Sargassum polycystum is light to dark brown in colour and is slightly coarse and sticky. It has a short and cylindrical stem with Y-shaped branches which can regenerate as vegetative propagules. These in turn produce primary laterals (branches) and phyllodes (leaves) which, developed into new thalli, but without a holdfast. In India, members of the genus *Sargassum* are amongst some of the largest perennial tropical seaweeds, growing abundantly on rocks, boulders, or available hard substrata (Lyra *et al.,* 2021). Primary branches bear irregularly alternate secondary branches with numerous simple and Y-shaped proliferations. It is found along the coasts of Perumanal, Idinthakarai, Koothankuzhi, Manapad, Trichendur, Thoothukudi, Kilakkarai, Pudumadam, Mandapam and Pamban areas (Anbuchezhian *et al.,* 2015).

The growth and shape of seaweeds are also influenced by a wide range of biotic and abiotic factors, including grazing, temperature, salinity, wave action, light, and light intensity. Although these variables interact to influence algal morphology, a few studies have reported how different environmental variables affect seaweed morphology (Kim *et al.,* 2022). Table 4.1. depicts the taxonomy and morphological characteristics of the selected seaweeds.

4.1.2 Nutrient and Heavy Metal Profile of Underexploited Seaweeds.

i. Macronutrients Composition.

Edible seaweeds have shown to be high in essential vitamins and minerals, at levels that would augment a balanced diet if consumed regularly. Specifically, trace elements and minerals are abundant in seaweeds compared to terrestrial foodstuffs, The typical strong taste associated with seaweeds is allied with the many beneficial micronutrients they contain (CMFRI, 2015). Since they are categorized as vegetarian, their usage is versatile in many cuisines across the continents in many food products (Mišurcová *et al.,* 2014). Table 4.2. shows the macronutrient profile of the selected edible seaweeds.

Table 4.2. Macronutrient Profile of the Selected Edible Seaweeds.

Nutrients (per 100g)	*Ulva lactuca*	*Ulva reticulata*	*Gracilaria edulis*	*Sargassum polycystum*
Moisture (%)	10.24±0.02	10.54±0.05	8.39±0.04	7.15±0.02
Ash (g)	20.62±0.06	19.96±0.05	15.25±0.03	9.14±0.01
Carbohydrate (g)	25.81±0.12	22.52±0.06	17.15±0.05	19.43±0.04
Protein (g)	19.22±0.13	22.10±0.07	20.35±0.05	18.17±0.01
Fat (g)	2.13±0.1	2.18±0.02	3.27±0.04	1.18±0.04
Crude fibre (g)	3.35±0.01	4.54±0.18	3.92±0.04	4.14±0.02

Data expressed as mean ± standard deviation (n = 3).

Moisture

Results of nutrient analysis of the seaweeds as depicted in the table 4.1 project the moisture, ash, carbohydrate, protein, fat, and crude fibre, on a dry weight basis. *U. lactuca* (10.24%), and *U. reticulata* (10.54%), had approximately comparable mean moisture contents, while *G. edulis* (8.39%) and *S. polycystum* (7.15%) had slight differences in their means when compared. When evaluating the shelf-life and quality of seaweed-based probiotic beverage, it is essential to take their moisture content into account because it can speed up microbe growth (Parthiban *et al.,* 2018).

Total Ash

In the current study, *U. lactuca* (20.62g) had the highest level of ash content, followed by a slight difference in *U. reticulata* (19.96g), and subsequently in *G. edulis* (15.25g) and *S. polycystum* (9.14g). In a previous study, Sanchez Machado *et al.,* (2004) reported that the ash level of *G. edulis* ranged from 19.07±0.61 to 34.00±0.11. Higher ash level in seaweed was a key sign of the presence of minerals, wherein Patel *et al.,* (2020) showed comparatively higher results. Generally, the ash content of seaweeds is much higher than that of terrestrial vegetables (Matanjun *et al.,* 2008). Anionic carboxyl, sulphate, and phosphate groups in proteins and cell wall polysaccharides are excellent binding sites for metal retention in seaweeds (Davis *et al.,* 2003).

Total carbohydrates

The Total carbohydrate content in *U. lactuca* (25.81g) and *U. reticulata* (22.52g) do not exhibit much difference in their means when compared to *S. polycystum* (17.15g) and *G. edulis* (19.43g), which are comparatively low in carbohydrates when compared to the green seaweeds in the present investigation. Abirami and Kowsalya, (2011) reported the total carbohydrate content in *U. lactuca* and *U. reticulata* to be 22.5 g and 20.4 g respectively, which was similar to the results obtained in the study, which could be due to the geographical location from where the samples are collected.

Protein

All the four selected seaweeds have protein content ranging from 18.17g to 22.10g. The total protein content of *S. polycystum* (18.17g) was higher than the results of Patel *et al.,* (2020) (10.29± 0.02%) and other *Sargassum spp. S. polycystum* and *U. lactuca* (19.22g) is slightly in a lower range when compared to the values of *G. edulis* (20.35 g) and *U. reticulata* (22.10 g), discussed in ascending order. A study by Suresh Kumar *et al*., (2014) showed the presence of protein content from 12.69 to 23.61 g per 100g of dry algal mass. The protein content of brown algae is generally low (10 - 17g of the fresh weight), whereas higher protein contents were recorded in green and red algae on an average of 21-35 percent fresh weight (Yada *et al.,* 2004). Similar results were obtained in the present study. According to Henriques *et al.,* (2007), the maximum protein content is seen in some of the Rhodophyta and some green seaweed belonging to the genus *Ulva*.

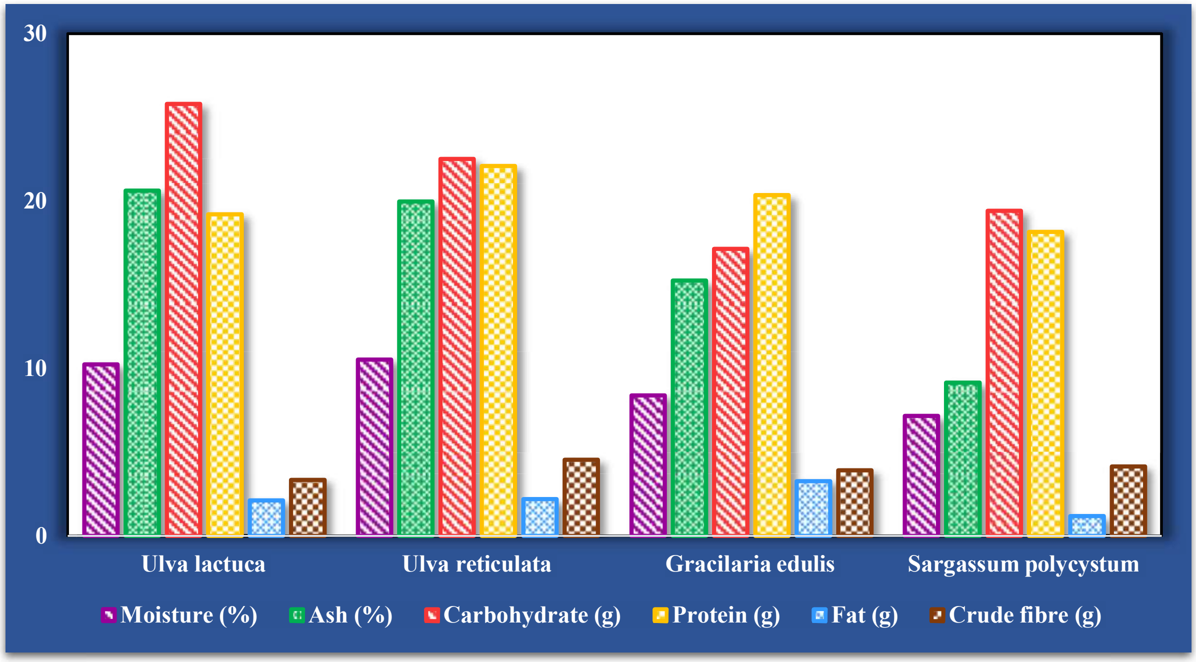
Total fat

The fat content of the selected seaweeds ranged from 1.18 g to 3.27 g. *U. reticulata* (2.18 g) and *G. edulis* (3.27 g) has slightly similar values and *U. lactuca* (2.13 g) and *S. polycystum* (1.18 g) had differences in the protein content. Fat content of *S. polycystum* was 1.18g which is lower than the previous research findings of 3.10 g. This study was in line with Norziah *et al.,* (2000) research and he stated that the differences in crude fat concentrations between various species of seaweed vary throughout the year and may be caused by their various growth stages, as well as by the seaweed's geographic location and climatic conditions said by Marinho-Soriano *et al.,* (2006). In general, despite the low-fat content, seaweeds are recognised as a rich source of biologically active lipids with a high proportion of unsaturated fatty acids, including n-3 Poly Unsaturated Fatty Acids (PUFAs) (MDPI, 2021).

Crude fibre

Seaweed species' varying growth stages and levels of photosynthetic activity, as well as seasonal changes in environmental factors that affect photosynthesis and nutrient intake, can all contribute to variations in the crude fibre of seaweeds (Siddique *et al.,* 2013). The study revealed that *U. reticulata* (4.54 g) had the highest crude fibre content among the selected seaweeds, followed by *S. polycystum* (4.14g), *G. edulis* (3.92 g), and *U. lactuca* (3.35 g). According to Wong and Cheung *et al.,* (2000) and Murugaiyan and Sivakumar *et al.,* (2008), dietary fibre is abundant in seaweeds and the soluble portion of dietary fibre demonstrates significant functional qualities like antimutagenic, antioxidant, and anticoagulant. Graph. 4.1. shows the proximate macro-nutrient content of the selected seaweeds.

Graph 4.1 Proximate Macro-nutrient Content of the Selected Seaweeds.



ii. Micronutrients Composition.

Ocean water contains a total of 92 minerals and vital trace elements. Seaweeds being constantly bathed in the oceans' nutrient-rich water naturally contain an optimum proportion of health-promoting minerals and vitamins (Burch, 2000). The vitamins and minerals composition of the selected four seaweeds are given in Table. 4.3. and Figure.4.2.

Studies reported that more than 30 percent of dry weight of marine algae is ash which contains various kinds of minerals (Chan *et al.,* 2020; García-Poza *et al.,* 2020).

Table.4.3. Micronutrient Composition of the Selected Seaweeds.

Nutrients (per 100 g)	*Ulva lactuca*	*Ulva reticulata*	*Gracilaria edulis*	*Sargassum polycystum*
Calcium (mg)	45±0.12	56±0.05	72±0.06	68±0.1
Phosphorus (mg)	148±0.02	186±0.19	210±0.05	165±0.04
Iron (mg)	68±0.06	42±0.05	37±0.19	32±0.03
Zinc (mg)	2.3±0.02	5.8±0.1	5.6±0.03	7.6±0.13
Vitamin C (mg)	46±0.04	32±0.03	39±0.13	42±0.03
β-Carotene (µg)	0.4±0.11	0.3±0.04	0.3±0.05	0.7±0.05

Data expressed as mean ± standard deviation (n = 3).

Calcium and Phosphorous

The highest concentration of calcium (72 mg) and phosphorous (210 mg) was recorded in *G. edulis,* whereas the lowest concentrations were found in *U. lactuca*, which has 45 mg of Calcium and 148 mg of Phosphorous respectively. The calcium content in other seaweeds is as follows: *S. polycystum* (68 mg), *U. reticulata* (56 mg), and *U. lactuca* (45 mg). The concentration of phosphorous was high in *U. reticulata* (186mg), next to *G. edulis,* followed by *S. polycystum* (165 mg) and *U. lactuca* (148 mg). Similar observations were recorded by Roleda *et al.,* (2015) and Wells *et al.,* (2017) in their studies.

Iron

The concentration of Iron ranged from 68 mg in *U. lactuca* and 42 mg in *U. reticulata,* 37 mg in *G. edulis* and 32 mg in *S. polycystum.* Maria *et al.,* (2010) reported that iron content in *Ulva spp.* was high about 60mg/100g, followed by *Sargassum spp.* 89.50mg/100g and *Gracilaria spp.* contained 36.60mg/100g of iron, which broadly correlated with the present study. The current investigation was also supported by a study by Thahira *et al.,* (2011), where the iron content of *U. lactuca* was higher in *U. reticulata*.

Zinc

The concentration of Zinc in *S. polycystum* (7.6 mg) was higher, followed by *U. reticulata* (5.8 mg)*, G. edulis* (5.6 mg) and *U. lactuca* (2.3 mg). Similar observations were noted by Hossain *et al.,* 2017. Kondaiah *et al.,* 2019 reported that zinc induces iron uptake and transcellular transport in intestinal cells via induction of DMT1 and FPN1 expression. Hence, zinc appears to be a key modulator of intestinal iron absorption and tissue iron distribution possibly mediated by regulating the DMT1 and FPN1 levels (Rolf *et al.,* 2021).

Vitamin C

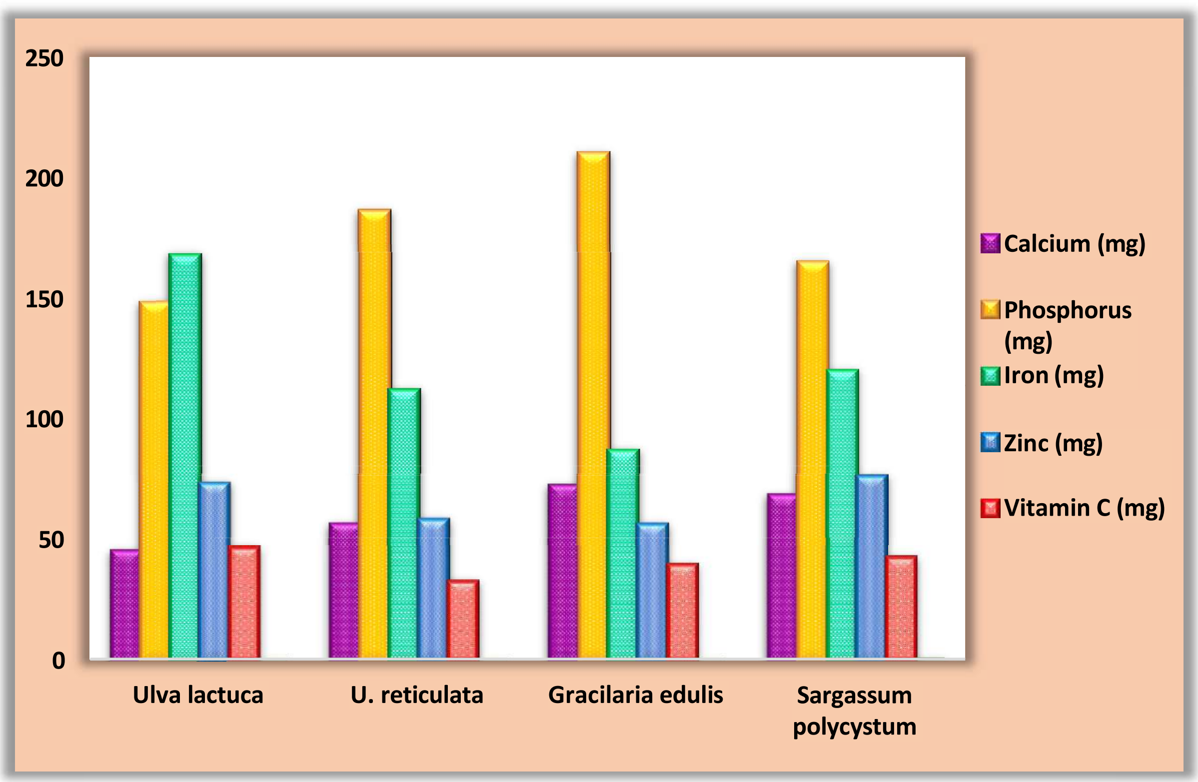
Vitamin C content was found to be considerably high in *U. lactuca* (46 mg), and lowest in *U. reticulata* (32 mg). *S. polycystum* had 42 mg and *G. edulis* had 39 mg. Several peer-reviewed papers state that seaweeds are a rich source of Vitamin C (Makkar *et al.,* 2016). Studies by Michalak *et al.,* (2014) reported a significant increase in the bioavailability of Iron in the presence of Vitamin C.

β-Carotene

In general, the carotenoid composition of green algae is similar to that of higher plants. According to Ookuzumi *et al.*, (1993) the main carotenoids present in green algae are lutein, beta-carotene, violaxanthin, zeaxanthin and neoxanthin. The current study reported 0.7 µg of β-carotene in *S. polycystum,* seconded by *U. lactuca* with 0.4 µg. It was found that both *U. reticulata* and *G. edulis* had 0.3 µg of β-carotene respectively. A study reported that marine algae are excellent sources of vitamins A, D, E, B-complex and B12 (Kolb *et al.,* 2004).

The composition and concentration of minerals differ based on the species and location since seaweeds can selectively absorb minerals from the ocean's surrounding saltwater and concentrate them in their thallus (Rohani-Ghadikolalel *et al.,* 2012). The current investigation observed that *U. lactuca* proved to be a considerably good source of Iron and Vitamin C, which is essential for incorporating it into a probiotic beverage for the *in vitro* iron bioavailability study using Caco-2 cell lines. Graph 4.2. gives the micronutrient composition of the selected seaweeds.

Graph 4.2. Micronutrient Composition of the Selected Seaweeds.



iii. Heavy Metals Composition.

Many marine organisms are known to accumulate and concentrate heavy metals several hundred times more than their concentration in seawater. The details of the heavy metal composition in the selected seaweeds on a dry weight basis are given in the Table.4.4.

Table.4.4. Heavy Metal Composition of the Selected Seaweeds.

Heavy metals (µg/100g of Dry weight)	*Safe limit	*Ulva lactuca*	*Ulva reticulata*	*Gracilaria edulis*	*Sargassum polycystum*
Mercury (Hg)	0.0016 mg	**BDL	0.006±0.02	0.007±0.02	0.13±0.02
Nickel (Ni)	79-105 µg	1.31±0.01	1.23±0.03	3.84±0.01	2.65±0.02
Lead (Pb)	0.025 mg	**BDL	**BDL	**BDL	**BDL
Cadmium (Cd)	0.07 mg	0.02±0.01	0.16±0.03	0.23±0.04	0.11±0.02
Chromium (Cr)	50 µg	0.42±0.01	0.29±0.03	0.36±0.01	0.55±0.02
		**BDL			

*The safe limit is as per the guidelines suggested by the World Health Organisation (WHO).

**BDL-Below Detectable Level.

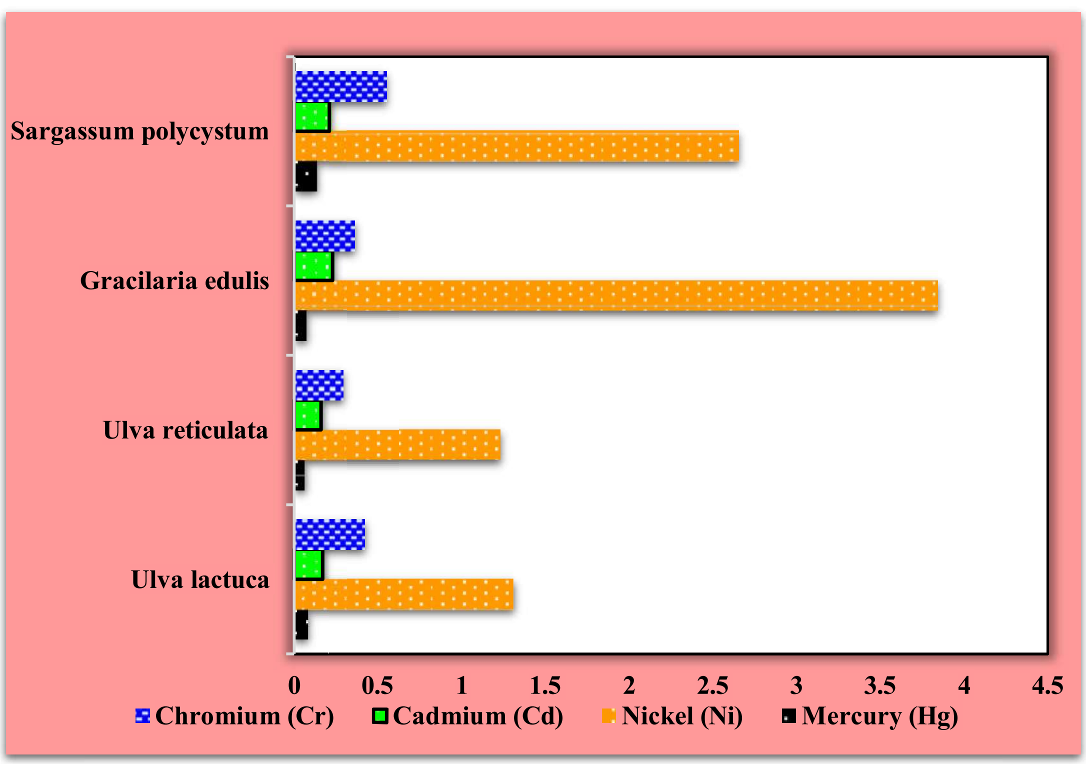
Data expressed as mean ± standard deviation (n = 2).

The results showed that *S. polycystum* (0.13 µg) had the highest concentration of Hg and *U. reticulata* (0.006 µg) had the least concentration. The concentration of Hg in *U. lactuca* is below the detectable level and significant differences were not observed in *U. reticulata G. edulis* (0.007 µg). All the seaweeds were shown to contain Ni, however, *G. edulis* (3.84 µg), had the highest concentration of Ni, which is also above the safe limit for consumption. *S. polycystum* (2.65 µg) had a marginal difference from the safe limit whereas concentrations of *U. lactuca* and *U. reticulata* were recorded to be 1.31 µg and 1.23 µg respectively. Lead and Arsenic concentrations were found to be below the detectable level. *U. lactuca* (0.17 µg), and *U. reticulata* (0.18 µg) were reported to have almost similar concentrations of Cd, whereas *G. edulis* (0.23 µg) and *S. polycystum* (0.21 µg) had concentration above the safe limit of consumption per day. Concentrations of Cr was found to be within the safe limit in *U. lactuca* (0.42 µg), *G. edulis* (0.36 µg), and *U. reticulata* (0.29 µg), whereas a slightly higher concentration was observed in *S. polycystum* (0.55 µg). Per the WHO and CEVA permitted values, Hg, Ni, Pb, and Cd were determined to be within legal limits in most of the observations, and *G. edulis* and *S. polycystum* exhibited concentrations of Ni and Cd beyond the safe limit.

Even though humans only require small amounts of the metal to manage a variety of bodily processes, including homeostasis stated by Naithani *et al.,* (2010) and Chandaka *et al.,* (2017), the consequences of prolonged consumption and heavy metal poisoning consist of nephrotoxicity, nasal and lung ulcers, skin ulcers, spinal/joint degeneration, immune system suppression, and lymphatic oedema, acute respiratory distress syndrome, lung cancer, pulmonary fibrosis, chronic bronchitis. (Das *et al.,* 2008).

According to the studies by Besada *et al.,* (2009) and Wells *et al.,* (2017), certain types of seaweed have a strong possession of heavy metals that are significantly affected by environmental factors such as salinity, and temperature, and pH fluctuations, photoperiods, accessibility, and concentrations of nutrients, etc. Heavy metal concentrations are reportedly high in seaweeds because of a significant amount of untreated effluent from numerous factories discharged into estuaries and coastal waters along the northern Bay of Bengal, which may have contributed to the contamination of seaweed with heavy metals (Chowdhury *et al.,* 2021). Graph 4.3. depicts heavy metal composition of the selected seaweeds.

Graph 4.3. Heavy Metal Composition of the Selected Seaweeds.



PHASE II

4.2 Sensory Acceptability of the Seaweed-incorporated Probiotic Beverage.

4.2.1 Organoleptic Scores of the Developed Beverage using 9-point Hedonic Scale.

The sensory evaluation was carried out to understand the organoleptic characteristics of the seaweed-incorporated probiotic beverage developed and which reflected in preference to consumption. The study subjects were familiarized with the scoring system to reflect consumer acceptability. The opinion of panellists indicated a good acceptance of the organoleptic properties of the product. The nine-point hedonic scale test was used for sensory acceptance, ranging from 1- dislike extremely to 9-like extremely. The parameters for assessment of the beverage included attributes like appearance, colour, consistency, flavour, taste, and acceptability of the beverage trial variations, as outlined in Table. 4.5.

Table. 4.5. Sensory Evaluation of Probiotic Beverage Containing *U. lactuca*.

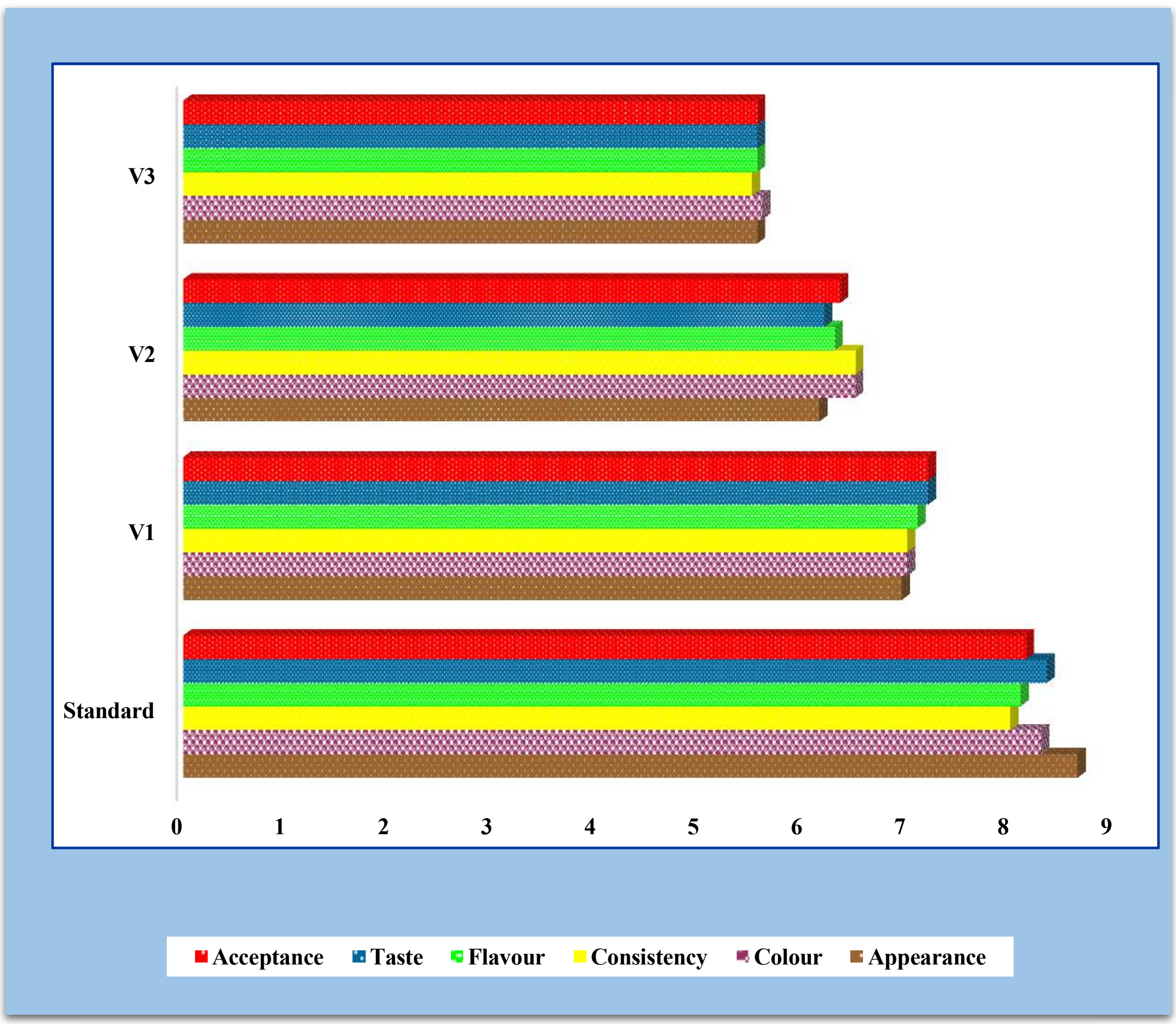
Variation	Appearance	Colour	Consistency	Flavour	Taste	Acceptance
Standard	7.65 ± 0.58^{ab}	8.3 ± 0.47^{a}	8 ± 0.56^{a}	8.1 ± 0.71^{a}	8.35 ± 0.58^{a}	8.15 ± 0.36^{a}
V_1	6.95 ± 0.51^{c}	7 ± 0.6^{ab}	7 ± 0.6^{ab}	7.1 ± 0.44^{abc}	7.2 ± 0.52^{abc}	7.2 ± 0.41^{abc}
V_2	6.15 ± 0.48^{ac}	6.5 ± 0.68^{ac}	6.5 ± 0.6^{ac}	6.3 ± 0.73^{ab}	6.2 ± 0.61^{ab}	6.35 ± 0.58^{abd}
V_3	5.55 ± 0.68^{bc}	5.6 ± 0.5^{abc}	5.5 ± 0.6^{abc}	5.55 ± 0.6^{ac}	5.55 ± 0.6^{ac}	5.55 ± 0.6^{acd}

Data are expressed as mean ± standard deviation (n = 20).
Different alphabets within the same column indicate significant differences across the formulations at $p \leq 0.05$

Statistical data on mean sensory scores comparison by ANOVA and Post–Hoc Tukey's test, obtained from preparation of probiotic beverage containing *U. lactuca* with varying formulations revealed a significant ($p<0.05$) difference between all three variations and the standard variant for each parameter.

From the above sensory assessment table, it can be inferred that the Standard beverage formulation scored the highest acceptance (8.15) and was extremely liked among the formulations. Variant 1 (V_1) scored the second highest (7.2) and was liked very much by the panellists. The lowest scores were awarded to the variants V_2 and V_3 and were less preferred by the panellists. A perusal of the study further revealed that the standard beverage overall scored high in terms of appearance (8.65), colour (8.3), consistency (8), flavour (8.1), and taste (8.35). V_1 score overall, the second highest score in terms of appearance (6.95), colour (7), consistency (7), flavour (7.1), and taste (7.2). V_1 was found to be liked moderately to very much, as per the sensory profiling. It is inferred from the study that the first variant's mean score was greater than the means of other established product variations, according to the findings of the sensory study. As a result, the first version of the beverage was picked for the standard nutrient analysis. The variants V_2 and V_3 were liked slightly by the panellists and hence were ruled out from further investigation based on sensory profiling. The low scores of Variants 2 and 3 could be attributed to the strong taste associated with seaweed. However, considering the health benefits associated with the ingredients used in the beverage, the scores were taken into consideration and further study was carried out. Graph 4.4. depicts the organoleptic scores of the developed probiotic beverage.

Graph 4.4. Mean Organoleptic Scores of Probiotic Beverage Containing *U. lactuca.*



PHASE III

4.3 Physical Characteristics, Nutrients and Nutraceutical Profile of the Developed Beverage.

4.3.1 Physical Characteristics of the Developed Beverage.

The determination of Physical characteristics like hydrogen ion concentration, total acid concentration, the content of dissolved solids in the beverage, and the relative density and flow of the beverage under the effect of gravity of the beverage were intrinsically related to their composition and structure (Sasue *et al.,* 2023). There is evidence suggesting that the health benefits of plant foods are attributed to the synergy or interactions between bioactive compounds and other nutrients in whole foods (Bochnak *et al.,* 2020). The table. 4.6. presents the physical characteristics of the developed probiotic beverage.

Table. 4.6. Physical Characteristics of the Developed Probiotic Beverage.

Parameters	Standard	Variant-I (V_1)
°Brix	18.25±0.07	18.78±0.03
Titratable acidity (%LA)	0.09±0.02	0.15±0.03
Viscosity (cP)	736.6	758.7
Specific gravity	1.24	1.36

Data are expressed as mean ± standard deviation (n = 3).

pH and titrable acidity in the beverage.

Variability of pH in food and beverage production can lead to critical differences in taste, freshness, and shelf-life of a final product (Torunoğlu *et al.,* 2017). Research suggests the pH range of 4-6±1.0 in probiotic beverages is acceptable since excess or low pH may hinder the quality and acceptability of the beverage (Bintari *et al.,* 2017 and Šertović *et al.,* 2019). It is observed from the table that the pH of Variant 1 is 6.43 and that of Standard is 6.52, both not varying much from the neutral pH of 7. Koirala *et al.,* (2011) also assessed the pH of fermented beverage developed from beets to be in the range of 6 to 7.5 which corroborated with the findings of our study.

Titratable or total acidity of fermented beverages is another crucial factor that indicates the sensory and microbial quality of the beverage and evidences whether the beverage is _balanced‘, how well it would age over time and whether it would be unacceptable or spoiled due to high level of acids produced by either bacteria or yeasts (Zoecklein *et al.,*2005). The analysis for total acidity calculated in terms of percentage equivalent of lactic acid disclosed moderate acidic levels present in the beverages. The results justify that with a decrease in pH, the total acidity increases as organic acids (citric, malic, succinic, lactic and tartaric acids) accumulate due to microbial activity (Kim *et al.,* 2010). The percentage values of titratable acidity for the developed beverage samples is 0.15 in Variant 1 and 0.09 in Standard beverage, as inferred from Table 4.6 and graph 4.5.

Total Soluble Solids (TSS) in the beverage.

Brix value indicates the amount of total soluble solids in the beverage. The total soluble solids (TSS) play a pivotal role in preservation and also affects the flavour of the beverage. V1 possesses 18.78±0.03°Brix when compared to the standard beverage, which contains 18.25±0.07°Brix. A study by Pimentel *et al.,* (2019) and Reale *et al.,* (2021) reported an association between TSS, Vitamin C and pH, during the storage period of the probiotic beverage. The TSS increased during the storage period and was reported to be increased by 2.25°Brix. Vitamin C and pH, decreased as the storage time increased. This could be attributed to the presence of Lactic Acid Bacteria (LAB) in the food product resulting in lower pH and thus increasing the titratable acidity value.

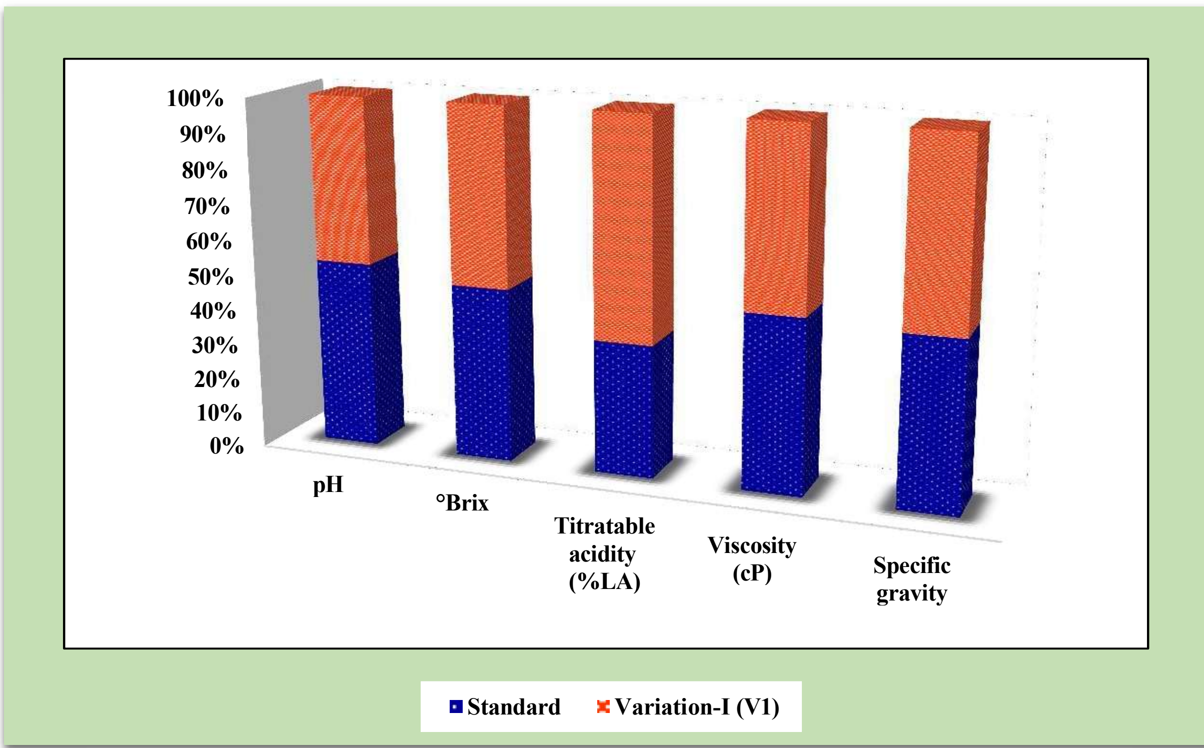
Viscosity.

Viscosity plays a significant role in determining the stability and uniformity of beverage suspensions by preventing sedimentation, throughout the product's shelf life (Shiby *et al.,* 2018). Considering the temperature-dependent nature of viscosity, understanding its variations under different conditions is vital for maintaining the quality and mouth feel of the beverage (Nguyen *et al.,* 2020). Viscosity is expressed as centipoise (cP) and is measured as millipascals (mPa). Variant 1 exhibits a viscosity of 768.7 cP whereas the standard has a value of 736.6 cP. Viscosity was increased in Variant 1 due to the presence of LAB and seaweed concoction, but it did not have any deleterious effect on other nutritional parameters.

Specific gravity.

Specific gravity measurements facilitate the precise formulation of ingredients, such as sugars and alcohol, crucial for maintaining batch consistency and accurately monitoring fermentation processes in beverages (Guimarães *et al.,* 2018). Additionally, specific gravity influences the beverage's density, affecting its mouthfeel and overall sensory experience (ref). The specific gravity of the beverage was determined to be 1.36 for Variant 1 and 1.24 for Standard beverage respectively (Ewe *et al.,* 2019). Graph 4.5. portrays the physical characteristics of the developed probiotic beverage.

Graph 4.5. Physical Characteristics of the Developed Probiotic Beverage.



4.3.2 Nutritional and Nutraceutical Profile of the Developed Beverage.

i. Proximate Nutritional Composition of the Developed Beverage.

Recent studies underscore the critical importance of the nutritional composition of beverages in promoting overall health and well-being (Ahmad *et al.,* 2021). The precise estimation of nutritional parameters in probiotic beverages is critical for validating their health benefits and ensuring product efficacy (Marco *et al.,* 2021). Nutraceuticals in probiotic beverages represent a burgeoning field of interest due to their potential health benefits, as reported by Shahidi *et al.,* 2020. The incorporation of nutraceuticals, such as vitamins, minerals, antioxidants, and bioactive compounds, into probiotic beverages can enhance their therapeutic efficacy, offering a synergistic effect that promotes gut health, boosts immunity, and reduces the risk of chronic diseases (Sanders *et al.,* 2014; Ouwehand *et al.,* 2002).

The proximate nutrient composition of 100 mL of the developed probiotic beverage is given in the table.4.7, and graphical representation is provided in Graph.4.6.

Table. 4.7. Nutritional Parameters of the Developed Probiotic Beverage (per 100mL).

Name of the compound	Standard	Variation-1
Total carbohydrate (g)	6.7±0.3	6.8±0.04
Reducing sugars (mg)	0.75±1.23	0.68±0.89
Crude protein (g)	0.98±0.03	0.79±0.02
Crude fat (g)	1.2±0.23	0.37±0.41
Crude fibre (g)	0.6±0.03	0.8±0.03
Dietary fibre (g)	0.1±0.02	0.2±0.02
Energy (kcal)	196±2.52	192.81±3.1
Iron (mg)	8.3±0.07	12.8±0.07
Zinc (mg)	3.1±0.03	4.2±0.02
Calcium (mg)	1.9±0.02	2.2±0.04
Sodium (mg)	2.6±0.05	2.6±0.03
Potassium (mg)	42.7±0.09	47.4±0.05
β-carotene (µg)	3.2±0.02	3.4±0.04
B_9 (µg)	20.1±0.01	29±0.05
B_{12} (µg)	1.2±0.05	1.3±0.02
Ascorbic acid (mg)	5.3±0.04	5.9±0.05

Data are expressed as mean ± standard deviation (n = 3).

Total carbohydrates.

Carbohydrates are one of the major macronutrients present in fermented beverages indicating metabolic enrichment during the fermentation process by various participating microorganisms in the starter culture. They are also quite useful in energy metabolism during fermentation as they can meet the energy requirement of the cell to a certain extent (Kardong *et al.,* 2022). Carbohydrates were quantified to be 6.8±0.04 g in Variant 1, with not much difference in the standard beverage with 6.7±0.3 g. The results have indicated that the amount of sugars present in the developed probiotic beverage mainly depended on the similar proportions of the ingredients used in the making of both variations of the beverage. These results were found to agree with the study by (Fadda *et al.,* 2017). These aforementioned facts reinforce our findings on total carbohydrates.

***Reducing sugars*.**

Estimating reducing sugars in a beverage involves quantifying the presence of sugars that possess free aldehyde or ketone groups capable of acting as reducing agents. Reducing sugars like glucose and fructose, serve as substrates for the fermentation of sugars into various metabolic products, including organic acids, alcohols, and other bioactive compounds by probiotic bacteria (Shen *et al.,* 2022). Fermentation efficiency and the metabolic activity of the probiotic cultures could hence be determined by assessing the sweetness and overall flavour profile of the beverage (Klayraung *et al.,* 2018). Variant 1 of the developed probiotic beverage contains 0.68±0.89 mg of reducing sugars when compared to 0.75±1.23 in the standard beverage.

Crude protein.

Understanding the protein content aids in quality control and product development, facilitating the formulation of beverages that can serve as viable sources of essential amino acids. Variant 1 contains 0.79±0.02 g and Standard contains 0.98±0.03 g of crude proteins, that could plausibly contribute to the presence of essential amino acids in the beverage. Study by Singh *et al.,* (2020). on Protein quality evaluation in probiotic beverages made from whey protein and soy protein, also reported 1.02 g of protein in developed beverages, which is comparable with the quantification of crude proteins in the current study.

Crude fat.

Probiotic beverages are known for their health benefits due to the presence of live microorganisms. Accurate determination of fat content is crucial for assessing the caloric value of the beverage. The crude fat content of Standard is 1.2±0.23 g/100mL and in Variant 1 it was determined to be 0.37±0.41 g per 100 mL of beverage. In the same line, (Tamang *et al.,* 2020) and (Jeyaram *et al.,* 2022) adjudged that the crude fat content of spirulina incorporated dairy beverage and white finger millet vegan probiotic beverage showed ~0.79 g/100 mL of crude fat, which is in line with the values obtained in the current study.

Crude fibre.

Fibre content in the diet is essential for maintaining several metabolisms and should be in a limitation to facilitate the bioavailability of essential nutrients in the body. The binding interactions between dietary fibre and iron, form complexes, thus reducing the solubility and absorption of iron in the gastrointestinal tract, as well as uptake by the enterocytes (Yung *et al.,* 2017). The crude fibre was found to be in less significant quantities, which is 0.8±0.03g/100 mL in variant 1 and with not much difference in standard with 0.6±0.03 g/100 mL. This proves to be a beneficial factor in iron bioavailability due to less or no hindrance in Iron uptake by the cells. In contrary to the readings observed in the current study, Barrangou *et al.,* (2018) on the Fermentation and functional properties of seaweed-based probiotic beverages, reported crude fibre content of ~12.5 mg/100 mL of beverage. One probable hypothesis for such discrepancy could be that the nature of the seaweed, and other ingredients, season and geographical location could play a major role in determining the nutritional composition (Correia *et al.,* 2017).

Dietary fibre.

Incorporating dietary fibre into probiotic beverages enhances their functional properties by serving as a prebiotic substrate, fostering the growth and activity of beneficial gut microbiota (Chen *et al.,* 2020). This interaction between dietary fibre and probiotics promotes gastrointestinal health by modulating intestinal microbiota composition and metabolic activities, thus potentially alleviating conditions such as constipation, diarrhoea, and irritable bowel syndrome (Almutairi *et al.,* 2019). Studies have demonstrated that dietary fibre, particularly soluble forms found abundantly in seaweed, can influence the absorption of iron due to its ability to form complexes with metal ions. This interaction may lead to either enhancement or inhibition of iron bioavailability, depending on various factors such as fibre type, concentration, and food matrix. For instance, soluble fibres can chelate iron ions, forming insoluble complexes that reduce iron absorption, while insoluble fibres may hinder the access of iron to intestinal absorption sites (Xie *et al.,* 2017). The study reports dietary fibre content of 0.1±0.02 and 0.2±0.02 g/100mL of beverage.

Energy.

Energy estimation plays a pivotal role in determining the calorie content of the beverage and in a broader scenario, in addressing IDA-associated malnutrition in vulnerable populations (Qu *et al.,* 2021). The energy content as deduced from the table and graphical representation shows 192.81±3.1 kcal in Variant 1 and 196±2.52 kcal per 100 mL of beverage. Li *et al.,* (2021). reported similar energy values from a novel probiotic beverage made from seaweed extracts.

Iron and Zinc.

Adequate iron intake is crucial for maintaining energy levels, cognitive function, and immune response (Amakye-Ansah *et al.,* 2021). While iron fortification is crucial for preventing anaemia, excessive iron can inhibit zinc absorption, potentially leading to zinc deficiency (Babu *et al.,* 2019). Zinc plays an intricate role in the metabolic pathways that facilitate iron absorption, though its direct impact on enhancing iron uptake is less pronounced compared to other factors (Cheong *et al.,* 2020). Conversely, zinc's role in maintaining a healthy gut and its involvement in the synthesis of metallothioneins—proteins that can bind to various metal ions—helps in managing oxidative stress and inflammation, thus potentially creating a more favourable environment for iron absorption (Park *et al.,* 2018 and (Ganesan *et al.,* 2022). Zinc is found in good amounts which values to 4.2±0.02mg/100mL in variant 1, thus complementing the iron uptake. The iron content of the Variant 1 is 12.8±0.07 mg/100 mL. The study is in line with Ramesh *et al.,* (2017) who postulated that the probiotic beverage containing seaweeds demonstrated iron in the range of 2.1-18.9 mg/mL and zinc in the range of 1.0-9.3 mg/100mL.

Calcium.

Calcium is a critical nutrient for consumption, whose primary function is maintaining optimum bone health. The involvement of Calcium in numerous physiological functions, including muscle contractions and nerve signalling, underscores its importance in the human body. The calcium content of Variant 1 is 2.2±0.04 mg/100 mL, which could be sourced from using whey as a major ingredient in beverage preparation. Studies by Silva *et al.,* (2020) and Hossain *et al.,* (2021) have reported calcium content of ~3.2 mg/100 mL, in fermented dairy beverages with *U. reticulata* concoction which could underpin the composition present in the developed probiotic beverage.

Sodium and Potassium.

Sodium and potassium are critical electrolytes in beverages, particularly significant for individuals affected by anaemia and malnutrition. Sodium is essential for maintaining fluid balance and proper muscle function, whereas potassium is vital for cellular function and cardiovascular health. In individuals with anaemia, a balanced electrolyte intake aids in enhancing blood volume and oxygen transport efficiency, thereby improving energy levels and reducing fatigue (Banerjee *et al.,* 2018). For those suffering from malnutrition, adequate sodium and potassium intake supports essential metabolic processes and prevents complications such as muscle weakness and cardiac arrhythmias (Zhu *et al.,* 2020). Empirical evidence suggests that beverages fortified with these electrolytes can markedly improve the nutritional status and overall health outcomes of these vulnerable populations (Fitzsimons *et al.,* 2019; World Health Organization, 2020). The sodium and potassium content in Variant 1 is quantified to be 2.6±0.03 mg/100 mL and 47.4±0.05 mg/100 mL respectively and that in Standard was 2.6±0.05 mg/100mL and 42.7±0.09 mg/100mL respectively.

β-carotene.

Beta-carotene is a precursor to Vitamin A and enhances iron absorption and mobilization, thereby mitigating iron-deficiency anaemia, which is prevalent in malnourished communities. Research indicates that beverages fortified with beta-carotene can significantly improve serum retinol levels, supporting immune function and overall health (Gupta *et al.,* 2020). Furthermore, beta-carotene's antioxidant properties contribute to cellular protection and enhance the efficacy of dietary interventions aimed at reducing micronutrient deficiencies (West *et al.,* 2002; WHO, 2009). The amount of β-carotene is 3.4±0.04µg/100 mL in variant 1 whereas it is 3.2±0.02 µg/100 mL in the standard beverage.

Folic Acid.

Folic acid is a synthetic form of the water-soluble Vitamin B9 (Cordero *et al.,* 2019). Anaemia, often caused by folate deficiency, is characterized by reduced red blood cell production due to impaired DNA synthesis. A study by Bailey *et al.,* (2015) demonstrated that folic acid fortification reduced the incidence of folate-deficiency anaemia by 25%. The current study reported 29±0.05 µg/100 mL of Folic acid which could be correlated with the results demonstrated by Espinosa-Martos *et al.,* (2017), thus complementing the current study.

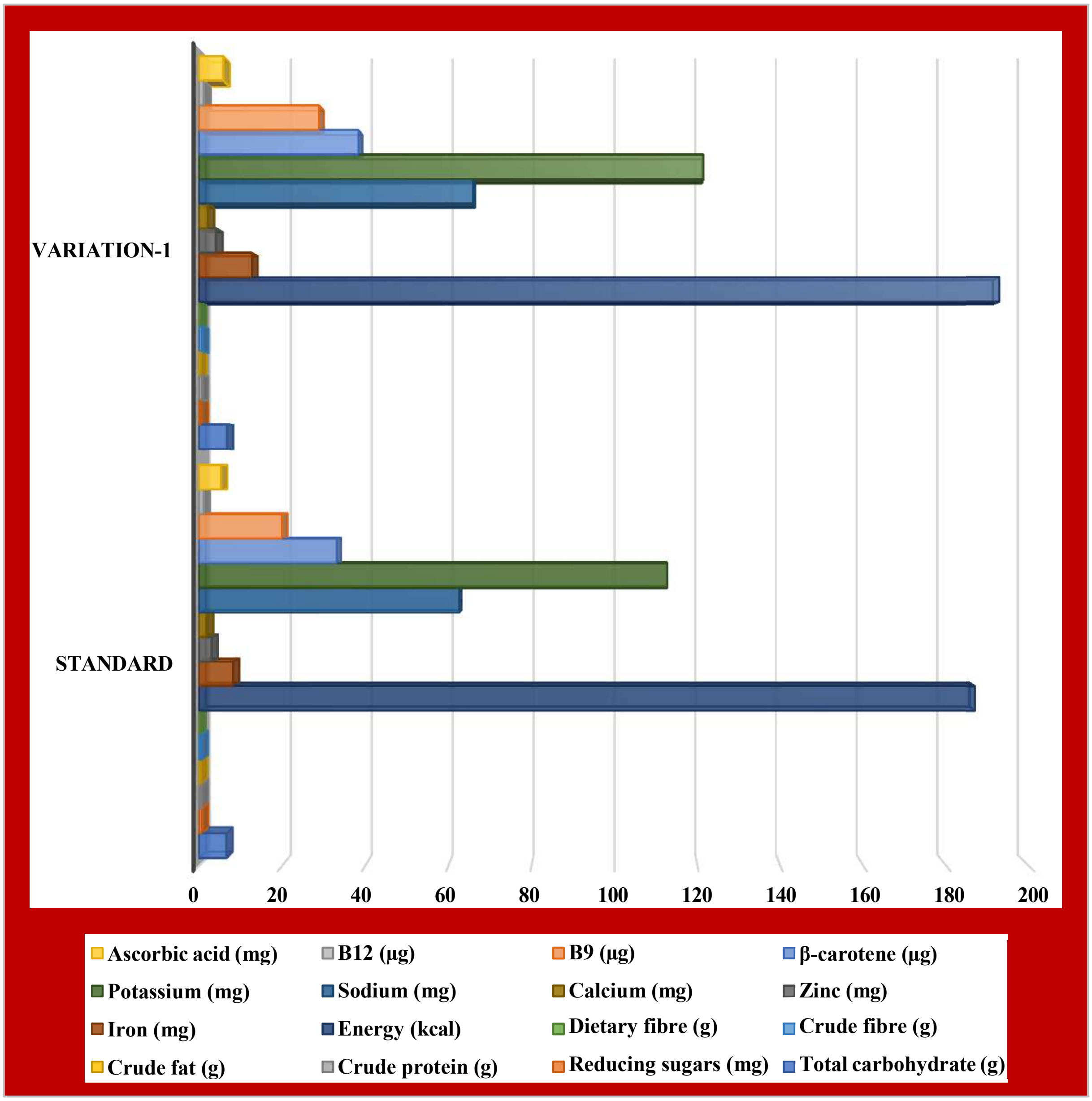
Cyanocobalamin.

As a vital micronutrient, Vitamin B12 plays a crucial role in hematopoiesis and neurological function. Anaemia, often caused by Vitamin B12 deficiency, can lead to fatigue, weakness, and impaired cognitive function. Moreover, malnutrition, especially prevalent in low-income populations, exacerbates the risk of anaemia and associated complications (Katre *et al.,* 2014) and Herrán *et al.,* 2019). The current study reported 1.3±0.02 µg/100 mL in Variant 1 and 1.2±0.05 µg/100 mL in standard, which ascertains that the beverage is a moderate source of Cyanocobalmin. Studies by Kumar *et al.*, (2016) and Ostadrahimi *et al.,* (2015) have reported ~1.8 µg/100 mL in their beverage.

Ascorbic acid.

Ascorbic acid, commonly known as vitamin C, plays a significant role in beverages aimed at addressing anaemia due to its facilitation of iron absorption. Vitamin C enhances non-heme iron absorption by reducing ferric iron to the more bioavailable ferrous form in the intestinal lumen, thereby increasing its uptake by enterocytes. Additionally, ascorbic acid helps counteract the inhibitory effects of phytates and polyphenols present in plant-based foods, further enhancing iron absorption (Garcia-Casal *et al.,* 2007; Hurrell *et al.,* 2010). Ascorbic acid was estimated to be 5.9±0.05 mg/100 mL in Variant 1 and 5.3±0.04 mg/100 mL in Standard, which could be majorly attributed to using orange juice in the beverage.

Graph 4.6. Proximate nutritional composition of the Developed Probiotic Beverage.



ii. Qualitative and Quantitative Phytochemical Profile.

Both qualitative and quantitative estimation of phytochemicals are crucial to assess the nutritional dynamics of the developed probiotic beverage and also aid in the validation of probiotic viability and functionality, ensuring that the probiotic strains deliver their intended benefits, such as improved gut health and immune support (Hill *et al.,* 2014). Table.4.8. records the estimated phytochemicals in the developed probiotic beverage.

Table.4.8. Estimation of Phytochemicals of probiotic beverage containing *U. lactuca* per 100mL.

Name of the compound	Standard	Variation-1	Standard	Variation-1
Phytates (mg)	+	+	18±0.02	24±0.02
Oxalates (mg)	+	+	20±0.03	21.5±0.02
Phenols (mg GAE)	+++	+++	62±0.03	68±0.03
Flavonoids (mg QE)	+++	+++	155±0.05	158±0.02
Alkaloids (mg)	+	+	5.4±0.03	7.2±0.03

Data are expressed as mean ± standard deviation (n = 3).

+++: Appreciable amounts; +Low to moderate amounts.

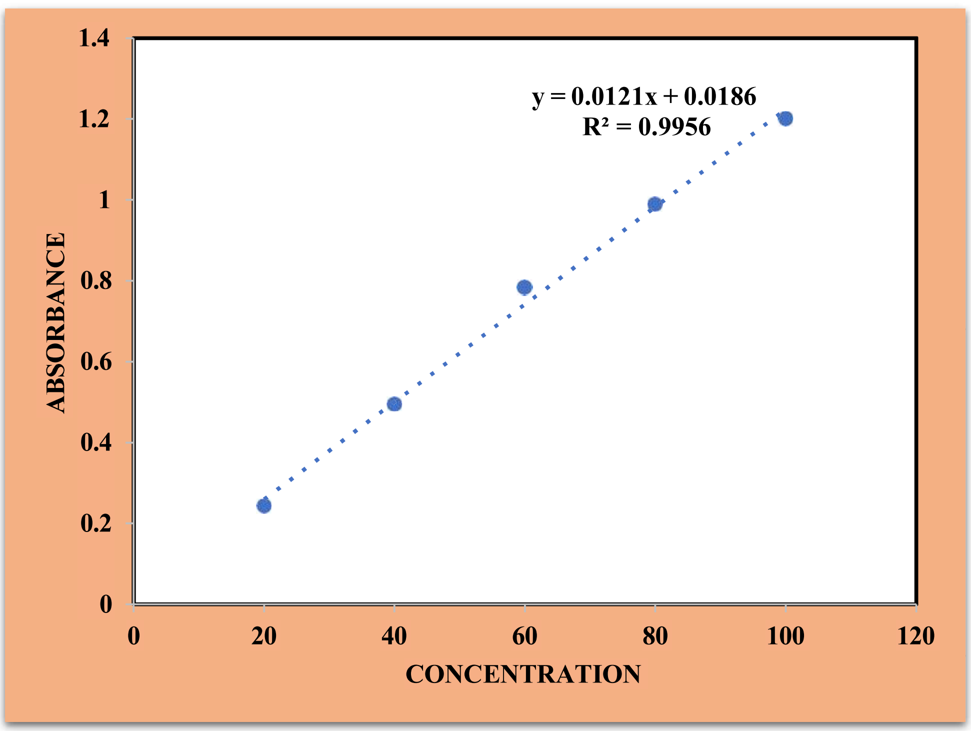
Phytates and Oxalates.

Phytates and oxalates are naturally occurring antinutrients found in various food sources like legumes, cereals, and seeds including edible seaweeds have been shown to influence iron absorption significantly. They bind to iron and form insoluble complexes, reducing its bioavailability (Baye *et al.,* 2017; Kumar *et al.,* 2017). Brown seaweeds such as *Ascophyllum nodosum* and *Laminaria digitata* have been reported to contain significant levels of these antinutrients (Parsons *et al.,* 2018; García-Casal *et al.,* 2016). However, the extent of inhibition of iron bioavailability by seaweeds can vary depending on the specific type of seaweed and also strategies such as fermentation and thermal processing have been explored to mitigate the adverse effects of these antinutrients, thereby enhancing iron bioavailability (Cofrades *et al.,* 2017 and Heaney *et al.,* 2018). The current study reported minimal quantities of phytates and oxalates in Variant 1 which was recorded to be 24.2±0.02 mg/100mL and 21.5±0.02 mg/100mL respectively. Standard beverage reported 18±0.02 mg/100mL and 20±0.03 mg/100mL of phytates and oxalates content respectively. The values reported are in line with the study by Chawla *et al.,* 2017 where the pearl millet (*Pennisetum glaucum*) derived probiotic beverage showed ~5 mg/100mL of phytates and ~18 mg/100mL of oxalates.

Total Phenols.

The total phenolic content in various edible seaweeds and seaweed drink matrices are noted for their high phenolic content, which contributes to their antioxidant properties and potential health benefits, including enhanced mineral absorption and metabolic health (Rajapakse *et al.,* 2016). Similarly, orange juice and whey used as ingredients in this beverage is rich in flavonoids and other phenolics, which can positively influence iron absorption by reducing the inhibitory effects of phytates and other iron-binding compounds (Patarra *et al.,* 2018; Kubola *et al.,* 2018). The interplay between phenolic content and iron absorption is complex, with phenolics potentially acting as both enhancers and inhibitors depending on their structure and the food matrix (Tsuda Król *et al.,* 2019; Król *et al.,* 2020). Tota phenolic content of the developed beverage was found to be 68±0.03 mg GAE/100mL in variant 1 and 62±0.03 mg GAE/100mL in Standard beverage variants. The current study bridges the ambivalent nature of phenolics in food and caters for a nuanced understanding of the importance of phenolics as bioactive compounds and their biochemical interactions to optimize the bioavailability of nutrients (Dey *et al.,* 2016; Hidalgo *et al.,* 2017; Granado *et al.,* 2021). Graph 4.7. depicts the Gallic acid standard curve used for the determination of the Total Phenolic Content of the developed beverage.

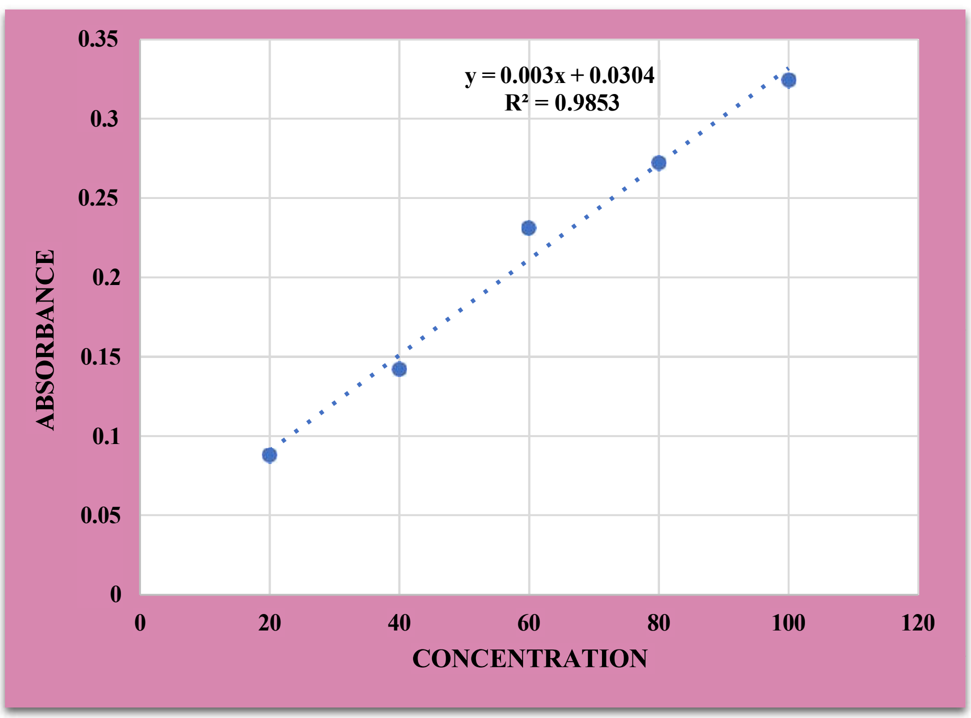
Graph 4.7. Gallic acid Standard Curve.



Total Flavonoids.

The total flavonoid content in edible seaweeds, orange juice, whey, and probiotic beverages has significant implications for iron absorption and enhancing their antioxidant properties. (MacArtain *et al.,* 2017). Orange juice, abundant in hesperidin, improves non-heme iron absorption by reducing ferric to ferrous iron (Eliason *et al.,* 2016). Whey, with bioactive peptides and flavonoids, positively influences iron absorption mechanisms (Sekse *et al.,* 2019). Probiotic beverages containing flavonoids and beneficial microorganisms, enhance iron bioavailability by modulating gut microbiota (Sazawal *et al.,* 2018). The synergistic effect of flavonoids and probiotics in these beverages optimizes iron absorption (Gibson *et al.,* 2019). Additionally, the antioxidant capacity of flavonoids reduces oxidative stress, aiding nutrient absorption (Pérez-Jiménez *et al.,* 2020). The findings of the current study recorded 158±0.02 mg QE/100mL of total flavonoids in Variant 1 and 155±0.05 mg QE/100mL in Standard beverages. A study by Egli *et al.,* 2019 reported ~589 mg QE/100mL of total flavonoids in Jabutical peel and fruit-based probiotic beverage containing *Gracillaria spp.* Extract. which highlights the nutritional and therapeutic potential of flavonoid-rich foods and beverages in addressing iron deficiency anaemia (Hurrell *et al.,* 2019). Graph 4.8. depicts the Quercetin standard curve used for the determination of the Total Flavonoid Content of the developed beverage.

Graph 4.8. Quercetin Standard Curve.



Alkaloids.

Probiotic beverages, which are increasingly consumed for their health benefits, have been identified to contain varying levels of alkaloids that could either enhance or inhibit iron uptake, according to the findings of Gómez-Guzmán *et al.*, (2018). For instance, research by Zhang *et al.,* (2017) demonstrated that seaweeds such as *Sargassum fusiforme* contain substantial amounts of alkaloids, which may influence iron bioavailability. Similarly, an analysis by Silva *et al.,* (2019) revealed that orange juice, while rich in vitamin C, also contains trace amounts of alkaloids that could potentially modulate iron absorption. Whey, a byproduct of cheese production, has been shown to possess alkaloids that might affect iron metabolism, as discussed in a study by Thompson *et al.,* (2020). A study by Reddy *et al.,* (2016) highlighted that certain alkaloids could form complexes with iron, thereby reducing its bioavailability. Conversely, research by Li *et al.,* (2021) suggested that some alkaloids might facilitate the reduction of ferric iron to the more absorbable ferrous form, thereby enhancing absorption. Furthermore, a comprehensive review by Patel and Singhal (2022) synthesized evidence from multiple studies and concluded that the net effect of alkaloids on iron absorption is highly context-dependent, influenced by the specific type and concentration of alkaloids present, as well as the overall composition of the diet.

The current study investigated the total alkaloid content of Variant 1 of the beverage to be 7.2±0.03 mg/100mL and that of Standard beverage to be 5.4±0.03 mg/100mL. A study by Rathod *et al.,* (2020) investigated the alkaloid content in newly formulated probiotic beverages using traditional herbs and spices. The findings indicated that these beverages contained significant levels of alkaloids such as piperine from black pepper and capsaicin from chili peppers. The incorporation of these alkaloids was found to not only enhance the flavour profile of the probiotic beverages but also contribute to their therapeutic properties, including improved digestion and anti-inflammatory effects. So far, relevant studies assessing the alkaloid content in seaweed-based probiotic beverage has not been studied to the best of our knowledge, hence the similarity study could not be quoted. These findings underscore the necessity of dietary interactions affecting iron bioavailability and suggest that further research is necessary to fully elucidate the role of alkaloids.

Correlation analysis between the sensory attributes and selected phytochemicals.

Correlation analysis was also carried out between the sensory attributes and select phytochemicals – ascorbic acid, total phenolics and total flavonoids, to understand the role of the latter in the sensory profile. Pearson correlation was carried out and the significant correlations are shown in Table.4.9.

Table. 4.9. Pearson Correlation coefficients for the relationship between Organoleptic scores and selected phytochemicals of the developed probiotic beverage.

	TPC	TFC	AsA	Acceptance	Flavour	Taste	Colour
Appearance	0.893*	0.839*	0.863	0.893*	0.972*	0.839*	0.928*
Colour	0.859*	0.886*	0.853*	0.883*	0.927*	0.962*	
Taste	0.998*	0.851*	0.962*	0.883*	0.949*		
Flavour	0.947*	0.998*	0.859*	0.977*			
Acceptance	0.896*	0.978*	0.893*				
AsA	0.954*	0.962*					
TFC	0.949*						

Pearson‘s correlation between appearance, colour, taste, flavour, acceptance, TPC, TFC and Ascorbic Acid is depicted in Table. Very strong correlations were observed between taste and TPC and Colour and TFC. (*r* = 0.998), followed by Acceptance and TFC (*r* = 0.978) Flavour and Acceptance (*r* = 0.977). Appearance and Flavour had a strong correlation of (*r* = 0.972). Ascorbic Acid and Taste; Ascorbic Acid and TFC and Colour and Taste had a similar correlation coefficient of (*r* = 0.962). Strong correlations were also observed between Ascorbic Acid and TPC (*r* = 0.954). TFC and TPC and TFC and Flavour correlated (*r* = 0.949). Appearance and Colour and Colour and Flavour demonstrated an *r* = 0.927 and 0.928 respectively. A strong correlation coefficient of (*r* = 0.896) was observed between acceptance and TPC. A correlation coefficient ranging from (*r* = 0.893-0.839) was observed between various combinations of sensory attributes and phytochemicals. All the values obtained were significant at $p \leq 0.01$ and $p \leq 0.05$ Hence, the role of various selected phytochemicals in determining the sensory attributes of the developed probiotic beverage was confirmed.

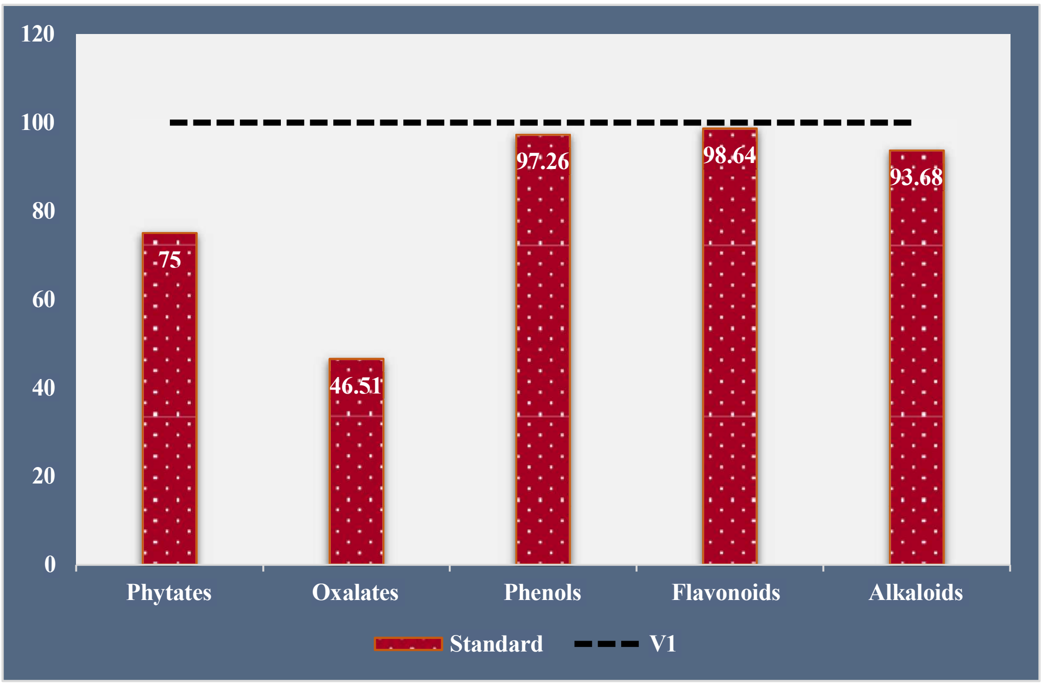
Phytochemical Quality Index.

Phytochemical Quality Index (PQI) is an essential parameter for the comprehensive evaluation of phytochemical quality content in the beverage. The individual phytochemical indices of the standard probiotic beverage to Variant 1, capped at 100, are depicted in the Figure. Since there is no Recommended Dietary Allowance (RDA) assigned to essential phytochemicals, there is an impending need to study the phytochemical quality ratios to determine the quality of phytochemicals in developed foods. The Overall Phytochemical

Composite Index (OPCI) of the standard beverage was calculated to be 94.89 and hence could be consumed at par with the *Ulva lactuca*-based probiotic beverage (V_1). As a further scope of the study, the PQI and OPCI of Variant 1 could be computed after assessing the RDA of the designated phytochemicals, estimated in the current study.

Micronutrient and Antinutrient Quality Score (MQS).

Graph 4.9. Phytochemical quality indices of Standard beverage compared to Variant 1.



In response to the increasing concerns about diet-health conditions, several systems have been set in place by scientists, food manufacturers and organisations to offer simple yet useful guidance to consumers to assist them in their food choices. Examples include the Index of Food Quality (Hansen, 1973); Naturally Nutrient Rich (NNR) score (Drewnowski, 2005); Nutrient-Rich Foods Index (Drewnowski, 2010); Overall Nutritional Quality Index (ONQI) (Katz *et al.,* 2010) etc. The micronutrient and antinutrient quality ratios are simple metrics to assess the percentage of RDA and the bioavailability of micronutrients in the developed probiotic beverage. These indices are useful to better understand the link between nutrition composition and their outcomes before making dietary recommendations (Cogill *et al.,* 2015). The phytochemical quality indices of standard beverage compared to Variant 1is depicted in graph 4.9. The mineral quality ratios of the Variant 1 and Standard beverage are given in Table. 4.10.

Table. 4.10. Individual Micronutrient Quality Ratios of the developed probiotic beverage.

		Micronutrient Quality Score (MQS)	
Nutrients	**RDA**	**Standard**	**V_1**
Calcium (mg)	800	0.24	0.28
Ascorbic acid (mg)	65	8.15	9.08
Iron (mg)	15	55.33	85.33
B_{12} (µg)	2	60.00	65.00
Potassium (mg)	2600	4.33	4.67
Zinc (mg)	11	28.18	38.18
B_9 (µg)	220	9.14	9.55
Oxalates (mg)	150	6.67	14.33
Phytates (mg)		1.80	2.40

As per National Institute of Nutrition nutrient content claims, food is categorised as a good source, if it contains 10-19 % of the DV of proteins/ vitamins/ minerals or dietary fibre. Food is considered an excellent source if it meets more than 20 % of the DV. High potency describes individual vitamins or minerals that are present at 100 % or more of the reference daily Allowance (RDA) per reference amount customarily consumed (NIN 2020). As per the Press Release of the Food Safety and Standards (Advertising and Claims) Regulations of India (FSSAI, 2018), a food is considered rich in protein if it meets 20 % of RDA per 100 g, a source of vitamin/minerals if it provides at least 15 % of the RDA per 100 g, high in vitamins/minerals if it provides at least 30 % of the RDA per 100 g, The results are discussed based on these guidelines.

A total of seven micronutrients and two anti-nutrients were assessed to determine the % RDA contribution of both beverage variants. It could be noted that 85.33% of RDA is met by Variant 1 whereas 55.33 % by Standard beverage. The difference in the values could be attributed to the use of *Ulva Lactuca* in Variant 1. The RDA of Vitamin B_{12} was 65% met by V_1 when compared to Standard beverage. 38.18% daily requirement of zinc was met by variant 1 when compared to standard beverage. A lower dietary requirement of Vitamin C was met by variant 1 viz. 9.08% and 8.15% by Standard beverage. This could be due to the usage of fresh orange juice which is naturally a good source of ascorbic acid. Both the beverages proved to be a poor source of calcium by contributing to only 0.24-0.28 % RDA. 4.67% and 4.33% of Potassium RDA requirements were met respectively by Variant 1 and Standard beverage. Phytates and oxalates were estimated to be 2.40% and 14.33% for Variant 1 and 1.80% and 6.67% respectively for the standard beverage.

iii. *In vitro* Antioxidant Potential of the Developed Beverage.

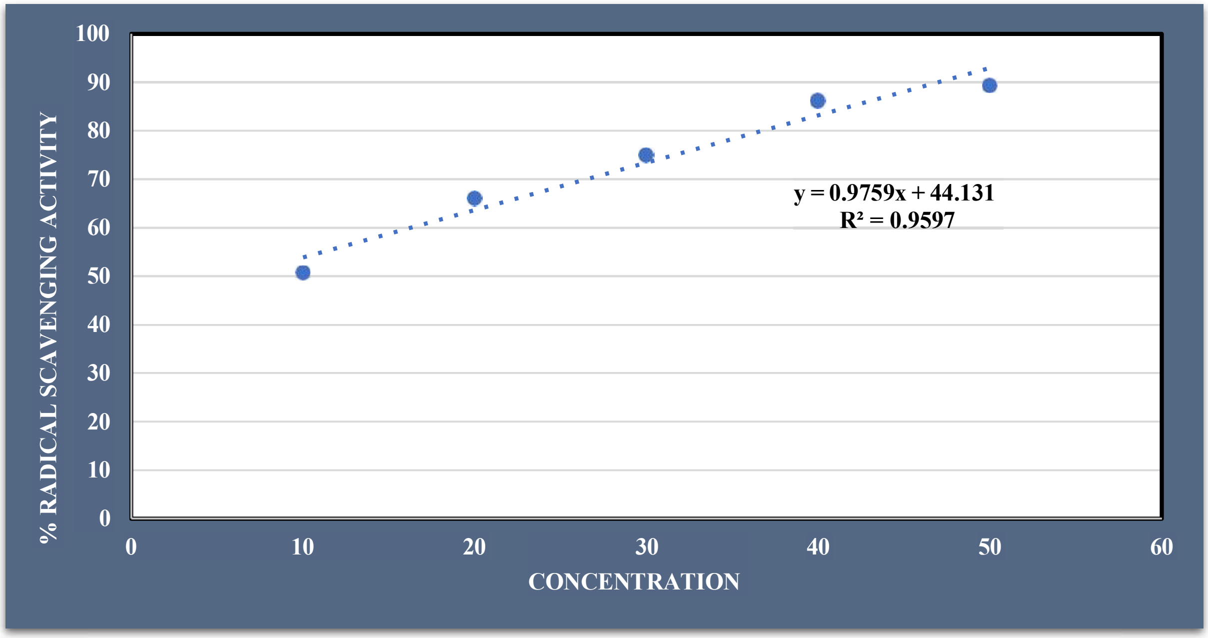
a. DPPH (Diphenyl-1-picrylhydrazyl) Radical Scavenging Assay.

The antioxidant activity based on the DPPH radical scavenging principle is where the DPPH accepts an electron to become a stable molecule. The methanolic solution of DPPH solution (violet) has a strong UV absorbance at 517 nm. The presence of a reducing agent in this methanolic solution pairs the odd electron of DPPH radical. The solution loses colour stoichiometrically and the absorbance decreases at 517 nm. Table. 4.11 depicts the percentage of Radical Scavenging Activity demonstrated by Ascorbic Acid, Standard beverage and Variant 1.

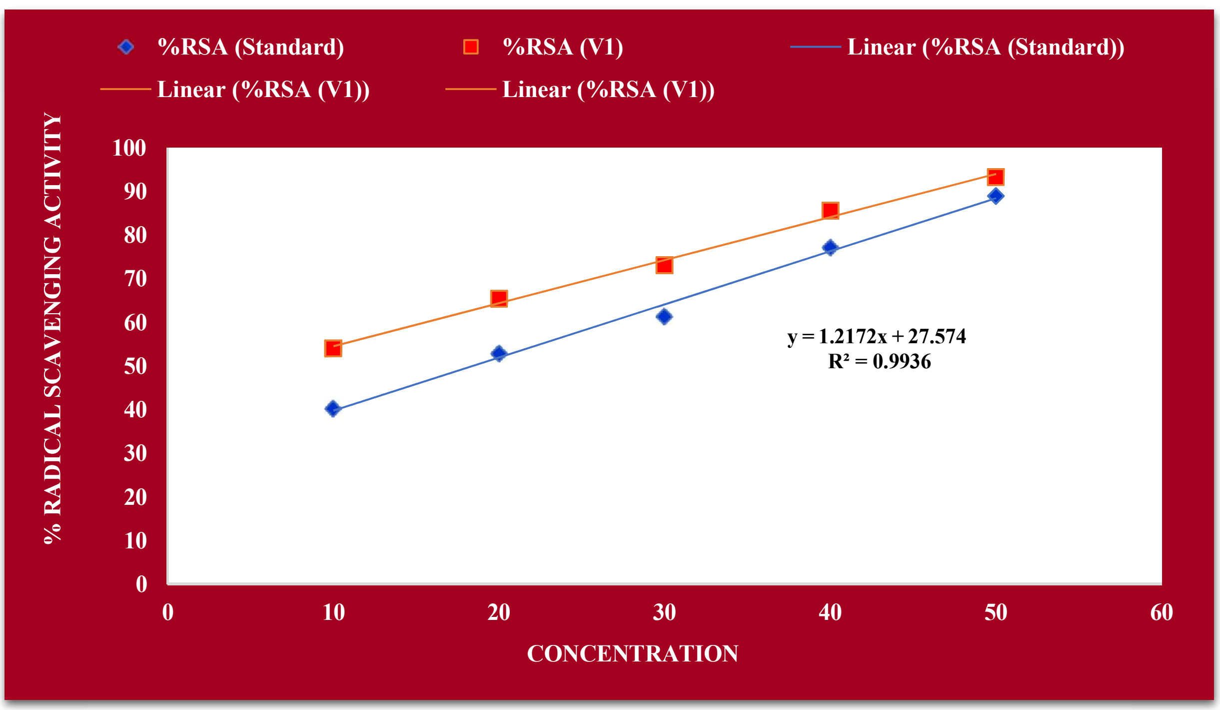
Table. 4.11. DPPH radical scavenging Activity of probiotic beverage containing *U. lactuca*.

Concentration (µg/mL)	%RSA		
	Ascorbic Acid	Standard	V_1
10	50.64	40.23	54.04
20	65.98	52.81	65.38
30	74.92	61.34	73.08
40	86.15	77.15	85.58
50	89.353	88.92	93.27
IC_{50} values	**6.01µg/mL**	**18.42 µg/mL**	**5.4 µg/mL**

Graph 4.10. (a). DPPH radical scavenging Activity of Ascorbic acid (positive control).



Graph 4.10. (b). DPPH radical scavenging Activity of probiotic beverage containing *U. lactuca*.



Graph 4.10 (a) and (b) shows the DPPH RSA of Ascorbic acid standard curve and that of the probiotic beverage. In the DPPH radical scavenging assay, antioxidants in the sample

extracts react with DPPH• by donating a hydrogen atom and converting it to a reduced form (Alam *et al.,* 2013). The degree of discolouration during this reaction indicates the radical scavenging potential of the sample. From the graph, it is evident that both Standard and Variant 1 of the developed probiotic beverage have shown very strong scavenging activity against DPPH. Variant 1 showed an exceptionally high DPPH RSA with an IC_{50} value of 5.4 µg/mL, which is higher than Ascorbic acid, with an IC_{50} value of 6.01 µg/mL, which was taken as a positive control for the study. The Standard beverage exhibited an IC_{50} value of 18.42 µg/mL, which is also commendable. With regard to the exceptional IC_{50} values demonstrated by the beverage, when treated with DPPH, it can be considered a very good source of antioxidants. Similar DPPH RSA of 72.59% was reported by Kumar *et al.,* (2019) by coffee beverage incorporated with *Sargassum whitti*, a brown seaweed. A study by Gomez *et al.,* (2023) also reported an IC_{50} value of ~0-15 µg/mL by a probiotic functional herbal drink developed from Indian goose berry (*Emblica officinalis* Gaertn.), which could be positively correlated with the current study.

b. Ferric reducing antioxidant potential (FRAP).

FRAP assay measures the ability of antioxidants to reduce ferric iron. Antioxidant molecules donate a single electron to the ferric-TPTZ complex, reducing it to a blue coloured ferrous-TPTZ complex. The higher the absorbance recorded, the higher the antioxidant activity. Variant 1 was found to exhibit 95.33±2 µmol Fe/g radical scavenging activity when compared to Standard, with 91.8±2.3 µmol Fe/g Radical scavenging activity. The table. 4.12. depicts the FRAP activity of the developed probiotic beverages.

Table. 4.12. FRAP activity of the developed probiotic beverages.

Radical Scavenging Activity (RSA)	Standard	V_1
FRAP (Ferric reducing antioxidant potential) (umol AAE/mL)	91.8±2.3	95.33±2

Correlation between phytochemicals and antioxidants.

The Pearson ‘s correlation between total phenolics, total flavonoids, total alkaloids, and antioxidant activities is depicted in Table. Very strong correlations were observed between total flavonoids and DPPH RSA (r = 0.985) followed by total flavonoids and FRAP

(r = 0.984), and total phenolics and total flavonoids (r = 0.949), Frap and DPPH RSA (r = 0.940) at $p \leq 0.01$. Strong correlations ($r > 0.8$) were also observed between total phenolics and DPPH RSA (r = 0.833) at $p \leq 0.01$. Negative correlation was observed between alkaloids and DPPH RSA (r = -0.835). No correlation was observed between Alkaloids, Antioxidant assays and other phytochemicals. Similar correlations were reported between DPPH and total phenolics (r = 0.882) and total flavonoids (r = 0.987) in non-dairy probiotic beverage developed finger millet (Hansa *et al.,* 2021). Another recent study by Jakubczyk *et al.,* (2020) also reported a similar positive correlation between total phenolics, total flavonoids, total anthocyanins, and antioxidant activity in fermented beverage Kombucha. Hence, the role of phenolics and flavonoids in the antioxidant activities of the developed probiotic beverage was confirmed. Table. 4.13. demonstrates the correlation relationship between Radical Scavenging Activity Assays and Phytochemicals assessed in the study.

Table. 4.13. Pearson Correlation coefficients for the relationship between RSA, TPC, TFC and alkaloid content of the developed probiotic beverage.

		FRAP	TPC	TFC
ALKALOIDS		**0.212**	**0.387**	**-0.572**
TFC		**0.984***	**0.949***	
TPC		**0.429***		
FRAP	**0.940***			

iv. Bioactive Compounds Profile using GC-MS/MS.

The developed beverage was characterized by GCMS (Model: QP 2020, Brand: Shimadzu). The identification of compounds was done by comparing with the data of NIST library. The attributes such as peak area, peak height, retention time (RT) and molecular weight are the basis of data in the NIST library. The retention time (RT), relative concentrations (peak areas %), compound name, molecular formula, and molecular weight (MW) of the components of the test materials were ascertained, the components identified by the GC-MS analysis are illustrated in Table. Results obtained from the gas chromatography-mass detector showed the presence of a high number of bioactive constituents in all tested fractions. This could give a clue to a wide medicinal activity they may possess. A total of 57 compounds were identified through GC-MS/MS out of which 13 compounds exhibited a match factor of >80% similarity with those established in the database. Graph 4.11. depicts a GC-MS chromatogram of the identified compounds. Table 4.14. depicts the Phytochemicals identified by the GCMS analysis of the developed probiotic beverage Variant 1.

Graph 4.11 GC-MS Chromatogram of the bioactive compounds identified in the study.

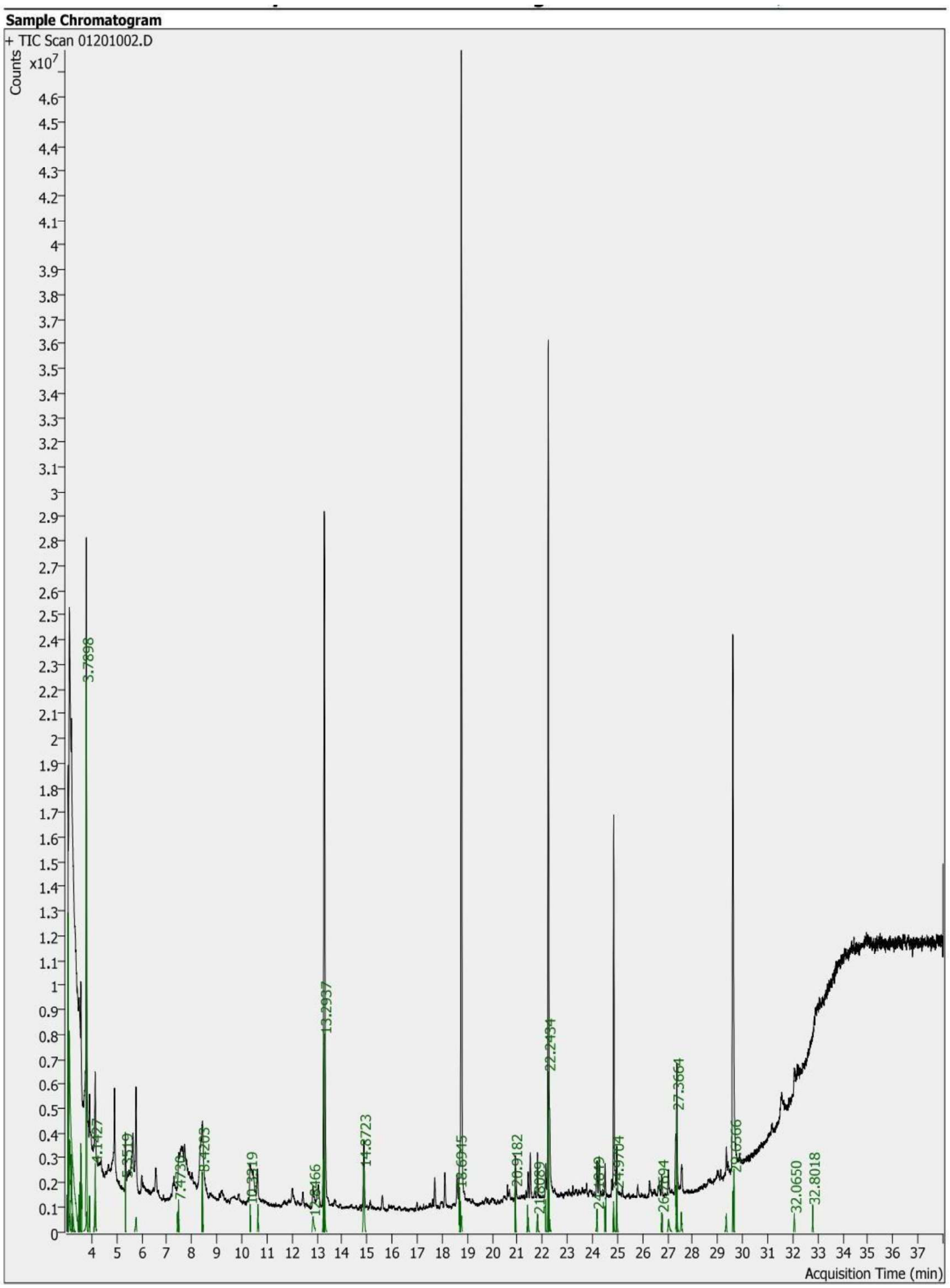


Table. 4.14. Phytochemicals identified by the GCMS analysis of the developed probiotic beverage Variant 1.

Sr. No.	Component RT	Compound Name	Component Peak Area	Biological Function
1	3.0634	1-Butanol, 2-methyl-	15576666.9	Flavors
2	3.1207	1-Propene-1-thiol	19096639.7	flavoring agent,
3	3.5674	Propanoic acid, 2-oxo-, methyl ester	6856070.9	Lower fatty acid, anti microbial agent
4	3.7898	Propionic acid	36439937.9	Pharmaceutical applications
5	3.7993	Ethanediamide, tetraethyl-	2099824.3	Anti cancer activity
6	3.9166	Propanoic acid, 2-hydroxy-, ethyl ester	1347897.1	Lower fatty acid, anti microbial agent
7	3.917	Methylal	1205263.2	Falvouring agent
8	4.1427	2-Ethoxypentane	5612628.9	Anti cancer
9	4.1478	2-Propanamine, N,N-dimethyl-	2700304.2	Used in drug manufacturing
10	5.3519	Acetic acid, 1-ethyl-2-methylpropyl ester	1288267	Ani microbial
11	7.4467	Tetraethyl silicate	1343632.4	As falvouring agent
12	8.4203	D-Limonene	3010396.9	dissolve cholesterol-containing gallstones.
13	8.4329	1,2,3,6-Tetrahydrobenzylalcohol, acetate	1250265.4	Essential oil
14	13.2906	8-Pentadecanol	8135304.2	antidiabetic, and anticancer activity
15	13.2937	Ethyl decanoate	5154900.9	antipyretic activity
16	18.6945	Octadecenamide	3647752.5	antimicrobial activity
17	18.7278	Decanoic acid, ethyl ester	1515057.3	Fatty Acid
18	20.9182	2,4-Di-tert-butylphenol	1887298.1	Anti fungal
19	21.4351	Pivalic acid, 3-fluorophenyl ester	1345817.1	Anti microbial
20	21.8089	Pentane, 1,5-diiodo-	1393267.7	Growth supplement for yeast
21	22.2434	Heptacosane	4165766.1	antiinflammatory activity
22	24.5397	Hexadecane, 1-iodo-	2591628.2	Anti microbial and anti oxidant
23	24.9704	Methyl hexadecanol	1617440.6	antiinflammatory activity
24	27.0448	Phthalic acid, pentyl tridec-2-yn-1-yl ester	1687866.3	Anti microbial

Sr. No.	Component RT	Compound Name	Component Peak Area	Biological Function
25	27.0448	Phthalic acid, bis(2-pentyl) ester	1602173	Anti microbial
26	27.3357	Hexadecanoic acid, ethyl ester	3731671.7	antioxidant, anti-inflammatory, hypocholesterolemic and cancer prevention activities
27	27.3664	Squalene	4003082.3	anti-oxidative and anti-inflammatory
28	27.5503	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-	1773741.1	Lower fatty acid, anti microbial agent
29	29.6566	Octadecanoic acid methyl ester	2293512.1	anti-oxidative and anti-inflammatory
30	32.8018	Isophthalic acid, 2-isopropoxyphenyl propyl ester 1000344-42-6	52.2	Anti microbial

Abundant Bioactive compounds.

Seven bioactive compounds exhibited abundance at retention times of 3.7898, 13.2937, 18.6945, 22.2434, 24.9704, 27.3664 and 29.6566. These were identified as propionic acid, ethyl decanoate, octadecenamide, heptacosane, methyl hexadecanol, squalene and octadecanoic acid methyl ester. These bioactive compounds were found to have therapeutic properties and were found to play a crucial role as antioxidant, anti-carcinogenic, ant-pyretic, anti-inflammatory, antibacterial, anti-allergic and also in ATPase generation. Table 4.15. and Figure 4.1. depict the GC-MS Chromatogram peaks of the abundant compounds in the developed probiotic beverage.

Table. 4.15. GC-MS Chromatogram peaks of the abundant compounds in the developed probiotic beverage.

RT	Compound
3.7898	Propionic acid
13.2937	Ethyl decanoate
18.6945	Octadecenamide
22.2434	Heptacosane
24.9704	Methyl hexadecanol
27.3664	Squalene
29.6566	Octadecanoic acid methyl ester

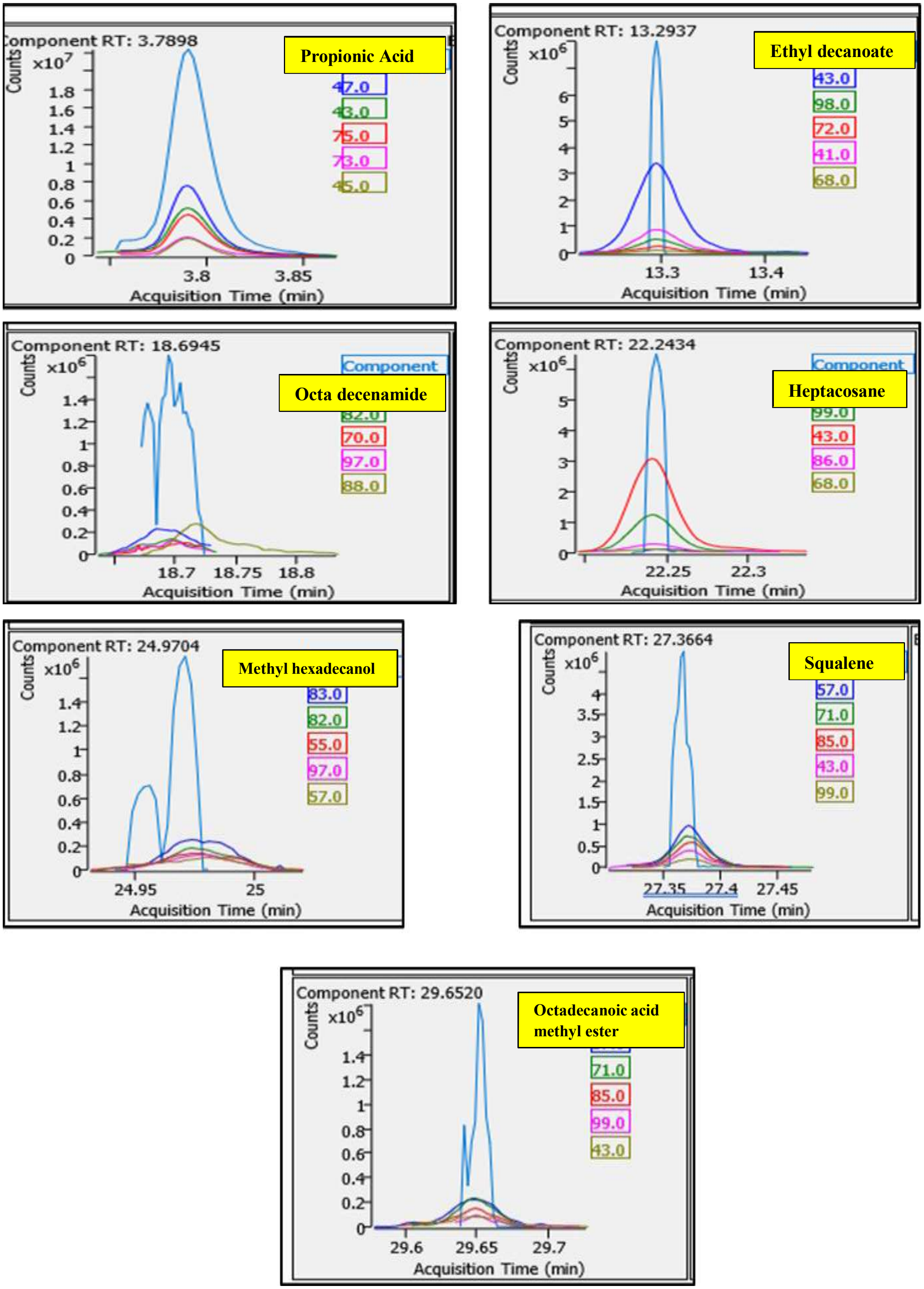


Fig. 4.1. GC-MS Chromatogram peaks of the abundant compounds in the developed probiotic beverage.

a. *In silico* ADME Profile of abundant Bioactive Compounds.

ADME screening was performed to assess the pharmacokinetics properties for the selected bioactive compounds using the QikProp module (Version 3.2, 2009, Schrödinger, LLC). for all the abundant bioactive compounds as depicted in the table.4.16. The Lipinski 's rule of five was further employed to evaluate oral absorption along with ADME.

The properties predicted were including log P (octanol/water), % human oral absorption in intestine (QP%) and predicted aqueous solubility in log S (molar), blood brain barrier (BBB), human intestinal absorption, Caco-2 permeable, number of likely metabolic reactions, number of property/descriptor values that fall outside the 95% range of similar values for known drugs and molecular weight which determines oral bioavailability. Table. 4.16 depicts the ADME properties of the abundant bioactive compounds from the developed probiotic beverage.

In silico ADME (Absorption, Distribution, Metabolism, and Excretion) profiling was performed to assess the pharmacokinetics properties for the selected bioactive compounds. Octanol/Water Partition Coefficient (Log P), indicates lipophilicity. Values between 1 and 3 are typically favorable for oral drugs. The compounds exhibited values between 3.829 in Squalene to 7.806 in octadecenamide, indicating moderate favourability. The percentage oral absorption indicated very good absorbability ranging from 80.359% in propionic acid, to 100% in ethyl decanoate, heptacosane, methyl hexadecanol, squalene and octadecanoic methyl ester. High permeability was predicted to be observed in Caco-2 cell model, with the log values ranging from 294.56 in octadecenamide to 9906.03 in squalene and heptacosane. Solubility (Log S) depicts the logarithm of the aqueous solubility and higher solubility (values closer to 0 or positive) is generally favorable for oral drugs. Propionic acid showed highest log value with 0.027 and low log values were observed for other compounds. Lipinski Rule of Five complied by all the abundant bioactive compounds and these compounds typically have optimal solubility and permeability, ensuring effective absorption and bioavailability. Compliance with these rules often results in better pharmacokinetic profiles, enhancing their potential as orally active drugs.

Table 4.16. ***In silico*** **ADME profile of Selected Bioactive Compounds.**

Compound	#stars	Donor HB	Accept HB	QPlog Pw	QPlog S	QPP Caco	QPPMDCK	#metab	Percent Human Oral Absorption	mol_MW	Rule Of Five
Octadecenamide	3	2	3	7.806	-6.902	294.565	146.042	2	93.019	281.481	0
Propionic acid	7	1	2.75	4.874	0.027	1384.156	702.999	1	80.359	74.079	0
Ethyl decanoate	1	0	3	4.076	-4.193	5399.811	3061.714	0	100	200.32	0
Heptacosane	12	0	0	4.696	-16.322	9906.038	5899.293	1	100	380.739	1
Methyl Hexadecanol	3	2	2	6.7	-9.052	4247.497	2362.042	1	100	256.471	1
Squalene	13	0	0	3.829	-14.424	9906.038	5899.293	1	100	410.725	1
Octadecanoic methyl ester	4	1	3	6.112	-8.695	5399.811	3061.714	1	100	296.492	1

PHASE IV

4.4 *In vitro* Probiotic Potential, Safety Assessment and Antimicrobial Activity of the Lactic Acid Bacteria in the Developed Beverage.

4.4.1 Morphological, Biochemical and Phenotypic Characterization of Probiotic Bacterial Strain in the Developed Beverage.

Morphological and Biochemical Characterisation of the Strain.

The morphological and biochemical characteristics of the isolated bacterial strain are elucidated in Table.4.17.

Table 4.17. Morphological and Biochemical Characterization of the *Lactobacillus* Strain.

Morphological characteristics		**Biochemical characteristics**	
MRS Broth	Mild to moderate turbidity	**Indole**	-
MRS agar	Irregular colonies	**Voges–Proskauer test**	-
Appearance and colour	White, creamy, and shiny colonies	**Methyl red test**	-
Type of colony	Small, clustered	**Oxidase test**	-
Ambient Growth temperature	25-40°C	**Catalase test**	-
Endospore test	Negative	**Citrate utilization test**	-
Gram reaction	Positive	**Carbohydrate fermentation**	
Motility test	Non-motile	**D-Glucose**	+
Shape	Elongated, rod-shaped, arranged in chains	**D-Fructose**	+
		D-Lactose	-
		D-Sucrose	+
		D-Xylose	+
		D-Maltose	-

The current study reported the presence of small, and clustered creamy white and shining colonies of bacteria with a mucilaginous appearance on the superficial plane of the agar medium, as depicted in Plate.4.2. The shape of isolates under the microscope (100x oil immersion objective) revealed long rod-shaped bacilli, confirming the gram-positive bacteria, as shown in the figure. Because Gram-positive cell walls have a thick peptidoglycan coating with multiple teichoic acid cross-links that resist decolourization, hence the colour of the cells remains purple (Deshpande *et al.,* 2017). The isolate showed positive under Gram staining and was dyed blue-purple in colour. The present study results coincide with commercial yoghurt where the gram staining was positive (Pyar *et al.,* 2014). Similarly, Akinola *et al.,* (2017) reported the shape of *lactobacillus* isolates under the microscope to be short rods in single or in clusters, long rods in single or network and cocci.

Plate. 4.2. Morphology of the LAB Isolate.

A distinctive sequence of growth was seen between the 25 and 40 °C temperature range. As depicted in Table. 4.17. the isolates exhibited negative outcomes for the tests' indole, Voges-Proskauer, methyl-red, endospore production, oxidase, catalase, and citrate utilization. Catalase negative bespeaks the ability of the isolates to not produce catalase enzyme (Akinola and Osundahunsi 2017). The isolates did not produce spores and were also non-motile. The hanging drop method showed the non-motility of the bacteria. It is one of the distinctive features of *Lactobacillus* where the flagella are absent. Similar results were observed in a study where traditional cheese and yoghurt in Iran were isolated and identified by biochemical tests where the motility showed negative, and the bacteria were non-motile in nature (Forouhandeh *et al.,* 2010). Catalase was assayed negative for Lactobacillus species. This could be substantiated by Mithun *et al.,* (2015) that the Lactobacillus shows negative in the catalase test because it could not interfere with the decomposition of H_2O_2 to deliver oxygen. According to the findings, the isolate(s) was/were capable of fermenting glucose, lactose, sucrose, fructose, maltose, galactose, and ribose. The findings of the study were found to be compatible with the results of other similar studies that used different seaweeds to develop fermented beverages (Arjun *et al.,* 2015; Phattayakorn *et al.,* 2013; Zhang *et al.,* 2016).

Phenotypic Characterisation of the Strain.

The generated BLAST results demonstrated that the 16S rDNA sequence of the isolated strain KYK demonstrates a high degree of similarity with species of *Lactobacillus* indexed in the GenBank and archived the accession number OP389067. The isolates showed the highest homology of 99% similarity index regarding species bacterial genera *Lactobacillus reuteri and Limosilactobacillus reuteri.* The phylogenetic tree in Figure. 4.2. represents the lineage analysis of the isolate with database sequences.

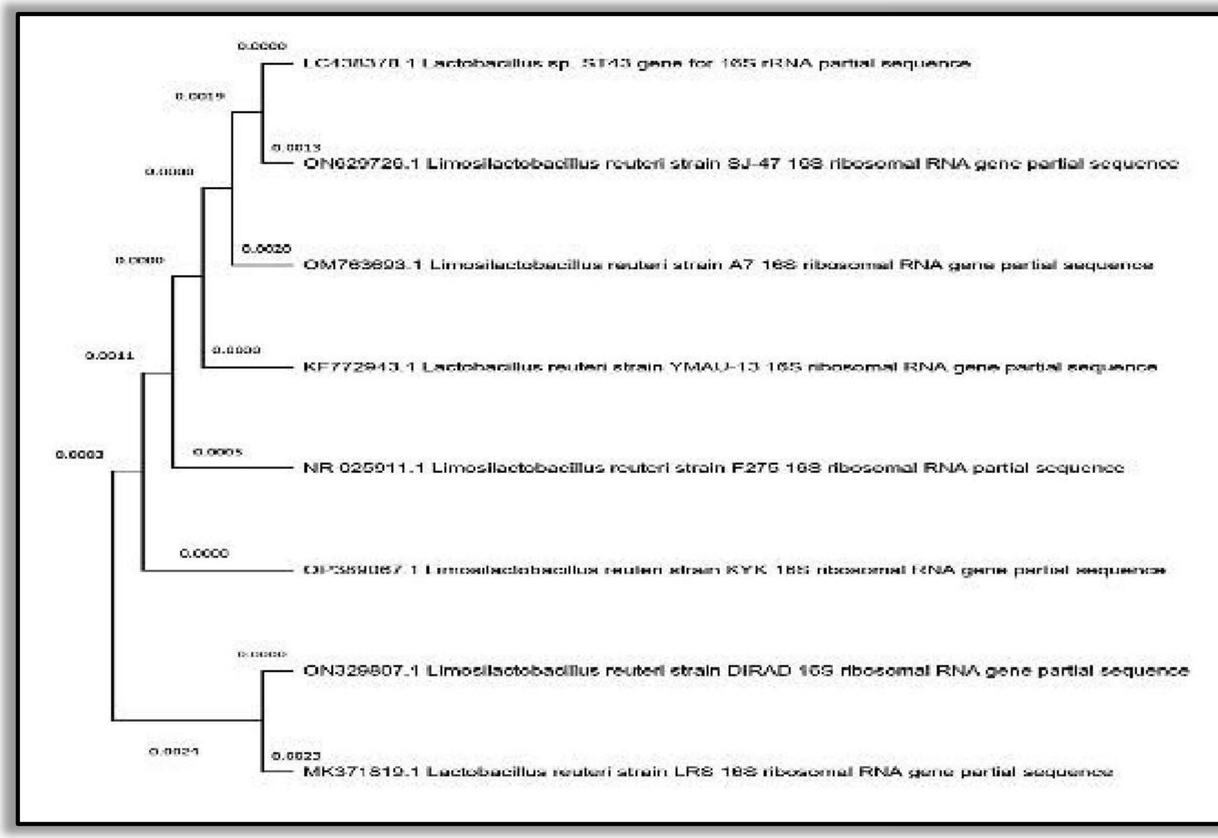


Fig. 4.2. Phylogenetic Tree of Isolate OP389067, depicting the Lineage Analysis of the Strain with the Database Sequence.

4.4.2 *In vitro* Probiotic Potential of Isolate OP389067.

Probiotics comprise Lactic Acid Bacteria (LAB) isolated from a variety of foods, but only those isolates with the best performance and high competitiveness are considered for use as probiotics (Somashekaraiah *et al.,* 2019). A probiotic isolate must possess characteristics like the capacity to survive as well as colonize under distinct environmental conditions (Palachum *et al.,* 2018). Gastric juice isolates should withstand bile salts but remain resistant to bile acids and adhere to epithelial cells despite the low pH. Moreover, they should have certain beneficial health properties, including antibacterial, anticancer, toxin-reducing, and anti-inflammatory properties. Therefore, *in vitro* evaluation is necessary to confirm that bacteria adhere to applicable surfaces and survive in the gastrointestinal tract before they are used as probiotics (Chiang *et al.,* 2012). The probiotic potential of OP389067 was assessed as per the parameters given below.

i. pH Tolerance.

Tolerance to pH is one of the essential attributes in in- vitro assays to determine the resistance to the acidic condition of the stomach. Probiotic microorganisms must demonstrate a surviving potential for an average time of 3h at pH $\leq$ 3 in the GI system. As the food remains in the stomach for at least 3 h (Thakkar *et al.,* 2015); this time limit was taken under consideration for in vitro assay. Table. 4.18. shows the total number of colonies at pH 3 and 7.2 for 0-3 hrs.

Table. 4.18. Tolerance to pH by Isolate OP389067.

Incubation time (h)	Total number of colonies OP389067 (Log_{10} cfu/mL)	
	3pH	7.2pH
0	7.2±0.1	7.6±0.07
1	7.3±0.02	7.8±0.03
2	7.5±0.04	8.2±0.02
3	7.6±0.02	8.5±0.02

From Table. 4.18. it is evident that there is a statistical association between the variables *L. reuteri* at pH 3.0 and pH 7±0.2 as the significance is 0.000, which is statistically significant (at 1% level). From the mean value, it is noted that sample Assay demonstrated more pH tolerance (41.70%) when compared to control samples. Figure4.13 (a) depicts the analogous growth of *L. reuteri* at pH 3.0 when collated with pH 7.2. The result of the study concerning the decrease in pH with an increase in fermentation time affirmed the reports by several previous studies substantially (Tamang and Thapa 2006; Chiang *et al.,* 2006; Kim *et* *al.,* 2013; Woldemariam *et al.,* 2014). The decrease of pH during the course of fermentation in our analysis could have been likely due to utilization of free sugars (Palaniveloo and Vairappan 2013) and generation of organic acids (e.g., lactic acid and other) during the course of microbial growth (Kyalakond *et al.,* 2006). In particular, Lactic acid bacteria (LAB) have been known to lower the pH by producing organic acids at the initial stage of fermentation preventing contamination by various other microbes since low pH is inhibitory to the growth of spoilage organisms but creates a competitive and conducive environment for the growth of the desirable organisms such as LABs (Palaniveloo and Vairappan 2013; Jang *et al.,* 2014).

ii. Simulated Gastric Juice Tolerance.

The viability of probiotic isolate is directly proportional to the tolerance against the gastric juice at pH 3. In table 4.19. Figure. 4.13 (b) shows that there is maintained growth (CFU/mL) of the *L. reuteri* isolate OP389067 at 3 pH when compared to the analogous increase in the growth of the organism at neutral pH. *L. reuteri* isolate OP389067 showed a survival rate of 99.4% after first-hour incubation. The viability decreased to 98.76% in the second hour and a marginal decrease was observed in the third hour, which is 98.25%. To find the association between sample Assay and Control (without gastric juice), Independent sample t-test results revealed that there was a statistical association established between the variables as the significance (p-value) value is 0.000, it was statistically significant (at a 1% level). From the mean value it is noted that *L. reuteri* isolate OP389067 at 3 pH demonstrated significant Simulated Gastric Juice Tolerance when compared to control at pH 7.2 without Gastric juice.

Table. 4.19. Tolerance to Simulated Gastic Juice by Isolate OP389067.

Incubation Time (h)	Total number of colonies OP389067 (Log_{10} cfu/mL)	
	3pH	7.2pH
0	9.65±0.01	9.23±0.05
1	9.64±0.005	9.34±0.04
2	9.63±0.01	9.41±0.02
3	9.61±0.01	9.61±0.01

iii. Bile Juice Tolerance.

The bile tolerance potential of the *L. reuteri* isolate OP389067 at 0.3% bile and its absence, determines the ability of the organism to break down lipids and fatty acids due to which viability of the bacteria diminishes (Hanif *et al.,* 2019). In Table 4.20., the statistical association between *L. reuteri* (3pH) and *L. reuteri* (7.2pH) presented that there is a significant association between the variables at 5% level, as the significance (p-value) value is 0.019. From the mean value, it is noted that *L. reuteri* with 0.3% Bile at pH 8 had more Bile Tolerance when compared to *L. reuteri* control. The *L. reuteri* isolate OP389067 showed a survival rate of 10.15% during 0-4h and with relation to absorbance, the isolate demonstrated a survival rate of 10.63% at 3 h, and there was considerable growth after 4h incubation time. Figure 4.13 (c) shows the survival rate of *L. reuteri* isolate OP389067 in 0.3% bile and in the control system.

Table.4.20. Tolerance to Bile Juice by Isolate OP389067.

Incubation Time (h)	Total number of colonies OP389067 (Log_{10} cfu/mL)	
	Assay	**Control**
0	9.16±0.05	9.23±0.05
4	8.23±0.05	9.56±0.05

iv. Pancreatin Tolerance.

The pancreatin tolerance of the *L. reuteri* isolate OP389067 for 0.5% Pancreatin concentration is determined by its ability to digest macronutrients with the help of the secreted enzymes from the pancreas. Table.4.21. gives the statistical association between *L. reuteri* isolate OP389067 at 0.5% bile concentration and the control illustrated a 5% significance as the significance (p-value) value is 0.015. From the mean value it is concluded that *L. reuteri* isolate OP389067 in 0.5% pancreatin had more Pancreatin Tolerance Absorbance at 600nm when compared to control. Figure 4.13 (d) show that there was a remarkable growth in the isolate at 24h in the presence of 0.5 percent pancreatin and the viability index of *L. reuteri* isolate OP389067 is calculated to be 25.33% at 24 h and 10.22% after 48 h incubation. Similar results were shown in the study by (Sharu *et al.,* 2016). that increasing the incubation time resulted in diminished viability of bacteria.

Table.4.21. Tolerance to Pancreatic Juice by Isolate OP389067.

Incubation Time (h)	Total number of colonies OP389067 (Log_{10} cfu/mL)	
	Assay	Control
0	9.48±0.01	9.50±0.01
24	15.51±0.01	16.81±0.01
48	14.58±0.01	15.28±0.01

Independent sample T-test was applied to portray the statistical significance across the in-vitro probiotic potential attributes, between the Assay and Control of *L.reuteri* isolate OP389067. Table. 4.22 represents the T-values and statistical significance between *L.reuteri* isolate 389067 at control and test pH.

Table. 4.22. Independent sample t-test depicting the statistical significance between *L. reuteri* isolate OP389067 and *L. reuteri* control.

Type of test	Variables	No. of colonies (Log10 cfu/mL)	T - Value	Significance
pH Tolerance	***L. reuteri* (3pH)**	7.6±0.05*	8.586	.000*
	***L. reuteri* (7.2pH)**	8.5±0.03		
Simulated Gastric Juice Tolerance	***L. reuteri* (3pH)**	9.63±0.21*	5.359	.000*
	***L. reuteri* (7pH)**	9.64±0.02		
Bile Tolerance	***L. reuteri* 0.3% Bile at pH 8**	8.70±0.03**	3.130	.019**
	***L. reuteri* control**	9.40±0.05		
Pancreatin Tolerance	**Assay (3pH)**	11.19±0.01**	.463	.015**
	Control (7.2pH)	13.87±0.02		

= Significant at 1% level** *= Significant at 5% level**

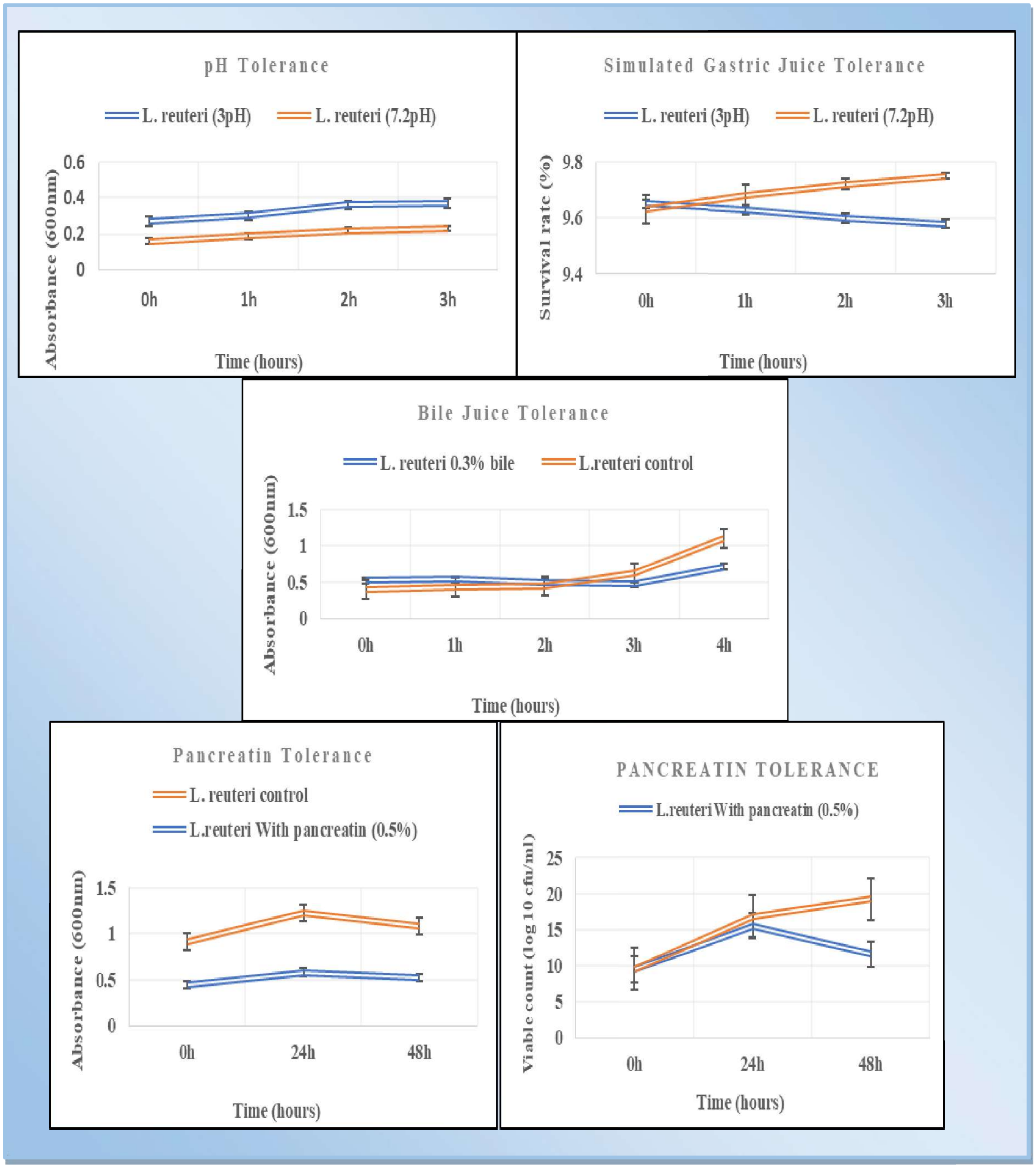


Fig. 4.3. Independent sample t-test depicting the statistical significance between *L. reuteri* isolate OP389067 and *L. reuteri* control.

(a): Growth of *L. reuteri* isolate OP389067 in pH 7.2 and pH 3.
(b): Survival rate of *L. reuteri* isolate OP389067 in pH 7.2 and pH 3.
(c): Survival rate of *L. reuteri* isolate OP389067 in 0.3% bile and in the control system.
(d): Survival rate of *L. reuteri* isolate OP389067 in 0.5% pancreatin and in the control system.

v. Cell Surface Hydrophobicity.

The potentiality of *L. reuteri* isolate OP389067 was determined by its ability to adhere to the intestinal epithelial cells. The isolate exhibited a remarkable 72.22% of adhesion for the n-hexadecane hydrocarbon. To maintain bacterial growth in the human GI tract, microorganisms must show adhesion to the intestinal epithelia, and this is likely made possible by the hydrophobic properties of microbes. Plate 4.3 and Table 4.23 demonstrates the cell surface hydrophobicity of isolate OP389067.

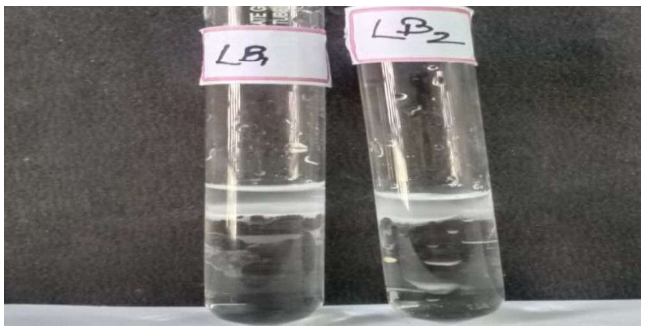


Plate.4.3 Cell Surface Hydrophobicity.

Table. 4.23. Cell Surface Hydrophobicity by Isolate OP389067.

Incubation Time (h)	Absorbance (600nm)
0	0.72±0.01
1	0.23±0.02

vi. Cellular auto-aggregation assay.

L. reuteri isolate OP389067 showed a significant 54.4% auto-aggregation thus exhibiting the potential to colonize and adhere to the intestinal epithelium. Table 4.24. shows the cellular auto-aggregation of isolate OP389067.

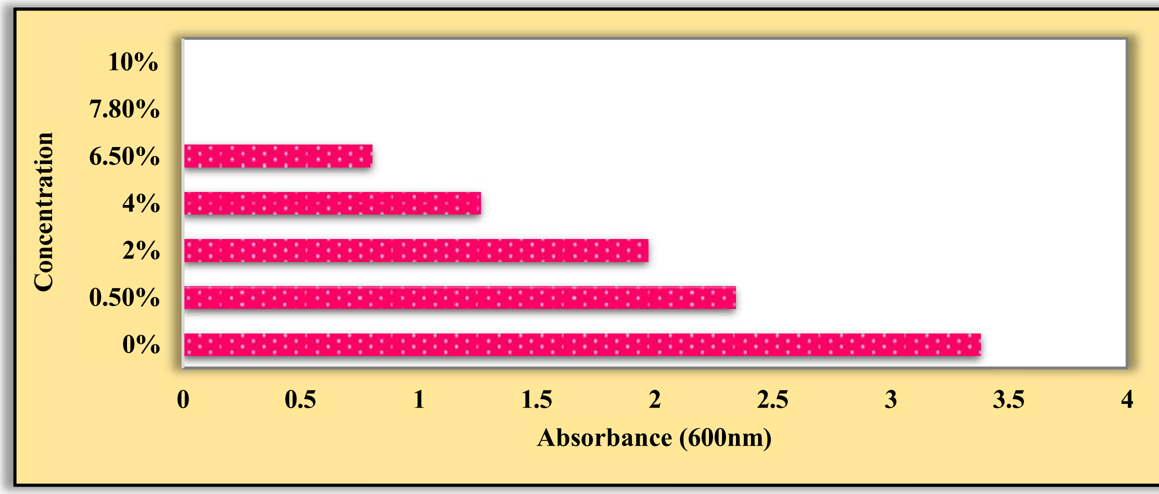
Table. 4.24. Cellular auto aggregation by Isolate OP389067.

Incubation Time (h)	Absorbance (600nm)
0	0.68±0.01
1	0.31±0.01

vii. NaCl Tolerance.

The rate of NaCl tolerance of *L. reuteri* isolate OP389067 is inversely proportional to the concentration of NaCl over 12 h duration.

Graph 4.12. Viability Index of *L. reuteri* Isolate OP389067 in Nacl at Different Concentrations.



Graph 4.12. shows considerable growth of the isolate until 6.5% concentration of NaCl and thereby absence of growth at and beyond 7.8% NaCl concentration. Tolerance to high salt conditions (6 - 8 percent) has been reported to be a distinctive feature in most species of lactobacillus (Carr *et al.,* 2002).

4.4.3 Safety Assessment and Antimicrobial Activity.

i. Haemolytic Activity.

L. reuteri isolate OP389067 showed γ-haemolysis or no haemolysis, which is a desirable characteristic of beneficial probiotic bacteria aiding in gut health.

ii. Antibiotic Susceptibility.

Based on the zones of inhibition, the LAB strains were designated as resistant (R), intermediate (I) and susceptible (S) as per Clinical and Laboratory Standards Institute guidelines [32]. *L. reuteri* isolate OP389067 was found sensitive to Amoxicillin sulbactam-AMS 30/15 mcg, Penicillin – P 10mcg, Amoxiclav– AMC 10mcg, and Chloramphenicol – C 30 mcg. Intermediate sensitivity was observed towards Gatifloxacin – GAT 5mcg and Moxifloxacin –MO 5mcg. However, *L. reuteri* isolate OP389067 was found to be resistant to

Methicillin – MET 5mcg and Ceftazidime – Cz 30mcg (Table 3). The association between the isolate and the susceptibility towards antibiotics was analyzed through One-Way ANOVA and statistical association between the variables as the significance (p- value) value is 0.000, is established at 1% level. Table. 4.25. gives the antibiotic susceptibility of *L. reuteri* isolate OP389067 against antibiotics at varied concentrations.

Table.4.25. Antibiotic Susceptibility of *L. reuteri* isolate OP389067 against Antibiotics at Varied Concentrations.

AK 30mcg	GAT 5mcg	MO 5mcg	AMS 30/15 mcg	MET 5mcg	P 10mcg	AMC 10mcg	C 30 mcg	Cz 30mcg
0.25±0.01	0.17±0.01	0.12±0.01	4.78±0.03	>250	5.2±0.1	6.8	5.96±0.05	>250
IS*	IS*		S**	R***	S**	S**		R***

*IS – Intermediate Sensitive; **S – Sensitive; ***R – Resistant

iii. Antagonistic Activity of the Extracted Antibacterial Agents.

The antagonistic activity of antibacterial agents in probiotic beverages is crucial for inhibiting pathogenic bacteria, thereby enhancing product safety and efficacy. Lactic acid bacteria (LAB) play a key role by producing organic acids, bacteriocins, and other antimicrobial substances that suppress harmful microbes, contributing to a balanced gut microbiota and overall health (Szulc *et al.,* 2022). Additionally, LAB-derived agents improve the shelf-life and safety of probiotic beverages by preventing spoilage and pathogen growth (Russo *et al.,* 2017; Chousalkar *et al.,* 2018). The antagonistic activity of *L. reuteri* isolate OP389067 and its antibacterial potential was assessed against *S.aureus, P.aeruginosa, B.cereus, S.typhi,* and *E.coli,* which are the most common enteropathogens affecting gut health. Plate 4.4 and table 4.25 depicts the Antagonistic Activity demonstrated by the extracted entibacterial agents of *L. reuteri* isolate OP389067.

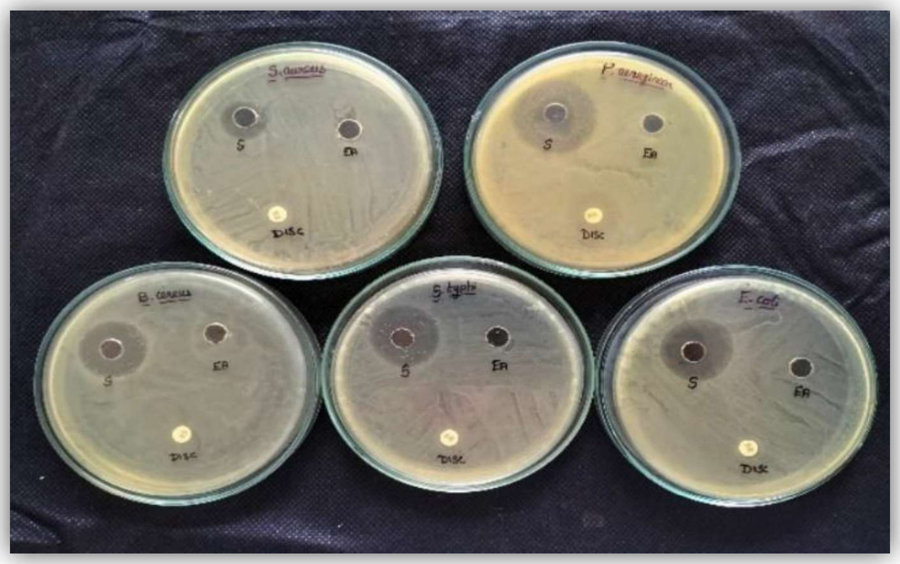


Plate. 4.4. Antagonistic Activity demonstrated by the Extracted Antibacterial Agents.

Table. 4.26. Antagonistic Activity demonstrated by the Extracted Antibacterial Agents of *L. reuteri* isolate OP389067.

	Zone of inhibition in mm				
	S.aureus	***P.aeruginosa***	***B.cereus***	***S.typhi***	***E.coli***
S- Sample	3±0.02	7±0.03	6±0.02	6±0.01	5±0.02
EA	Nil	Nil	Nil	Nil	Nil
Disc	Nil	Nil	Nil	Nil	Nil

S- sample (supernatant+ ethyl acetate; EA- ethyl acetate –negative control; Disc – antibiotic disc – Vancomycin 30mcg.

Table. 4.27. Minimum Inhibitory Concentration (MIC) of *L. reuteri* isolate OP389067 on Bacterial growth.

Name of the Bacterial Strain	Minimum Inhibitory Concentration (mg/mL)
Staphylococcus aureus	**1.5**
Escherichia coli	**1.6**
Klebsiella pneumoniae	**0.8**
Pseudomonas aeruginosa	**0.9**
Bacillus cereus	**1.2**

The isolate demonstrated moderate sensitivity towards *B.cereus* and *S.aureus* with a zone of inhibition of 10±0.01 and 11±0.01 respectively. Very low sensitivity was observed for *P.aeruginosa, S.typhi,* and *E.coli* with a zone of inhibition 8±0.05, and 9±0.02 towards both organisms respectively.

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent that prevents the visible growth of a microorganism. Table. 4.27 shows the Minimum Inhibition Concentration of *L. reuteri* isolate OP389067 on bacterial growth. The isolate exhibited an MIC of 1.5, 1.6, 0.8, 0.9 and 1.2 mg/mL by *S. aureus, E. coli, K. pneumoniae* and *B. cereus* respectively. *Lactobacilli* have been commonly found to produce antimicrobial substances that inhibit the growth of various pathogens like *S. typhi, E. coli, B. cereus, S. aureus, K. pneumoniae, P. aeruginosa, L. monocytogenes*. (Chowdhury *et al.,* 2012, Singh *et al.,* 2012, Amdekar *et al.,* 2010, Mallesha *et al.,* 2010, Danisco Technical Memorandum).

4.4.4 Antimicrobial Activity, Storage and Shelf-life Study.

The shelf-life study data of the developed probiotic beverage is elucidated in Table. 4.28. The amount required to gain any therapeutic benefits is a minimum of 106 viable probiotic cells per millilitre during storage until the expiry date.

Table.4.28. Shelf-life Study of Probiotic Beverage Containing *U. lactuca* at 4±2℃

Days	pH		Acidity %		Total Viable Count (cfu/mL)		Total fungal count (cfu/mL)		Total bacterial count (cfu/mL)	
	Std	V_1	Std		Std	V_1	Std	V_1	Std	V_1
0	6.9	7.0	0.02		1.82x10^{7}	2.79x10^{7}	Nil	Nil	Nil	Nil
2	7.2	7.3	0.04	0.09	2.02x10^{7}	3.08x10^{7}	Nil	Nil	Nil	Nil
4	7.8	6.9	0.09	0.15	2.28x10^{7}	3.36x10^{7}	Nil	Nil	Nil	Nil
6	6.9	6.5	0.23	0.20	3.12x10^{7}	4.12x10^{7}	Nil	nil	Nil	Nil
8	6.2	5.9	0.28	0.31	4.89x10^{8}	5.19x10^{8}	BDL*	nil	3.27x10^{7}	4.76x10 7
10	5.8	5.4	0.35	0.35	5.23x10^{8}	6.92x10^{8}	BDL*	BDL*	3.83x10^{8}	5.24x10

BDL* Below Detectable level

The cell viability of *L. reuteri* isolate OP389067 ranged from 3.36x10^{7} to 6.92x10^{8} which justifies the standard viable count of bacteria essential to cater to the therapeutic benefits of the developed probiotic beverage. The shelf-life study of the beverage at a refrigerator temperature of 4°C recorded the absence of fungus from0-6th day of storage duration. From day 8th-10th, fungal count was found to be below the detectable level for the standard beverage and the Variant 1. The total bacterial count for both standard product and the variant was found to be exponentially increasing from 3.27x10^{7} to 6.83x10^{8} and 4.76x10$^{\mathbf{7}}$ to 6.70x10$^{\mathbf{8}}$ for the standard and variant respectively. The pH of the product gradually decreased from the range of 7.0 to 5.0 for both standard and variant beverages within 10 days duration. The percentage acidity showed a spike in values for both standard and variant beverages for a span of 10 days. From the above data, it was noted that the shelf life of the studies of probiotic beverages containing *U. lactuca* at 4±2℃ was found to be best for consumption within 10 days of preparation at a refrigerator temperature of 4±°C. Plate. 4.5. depicts the fungal and bacterial growth observed during the shelf-life study.

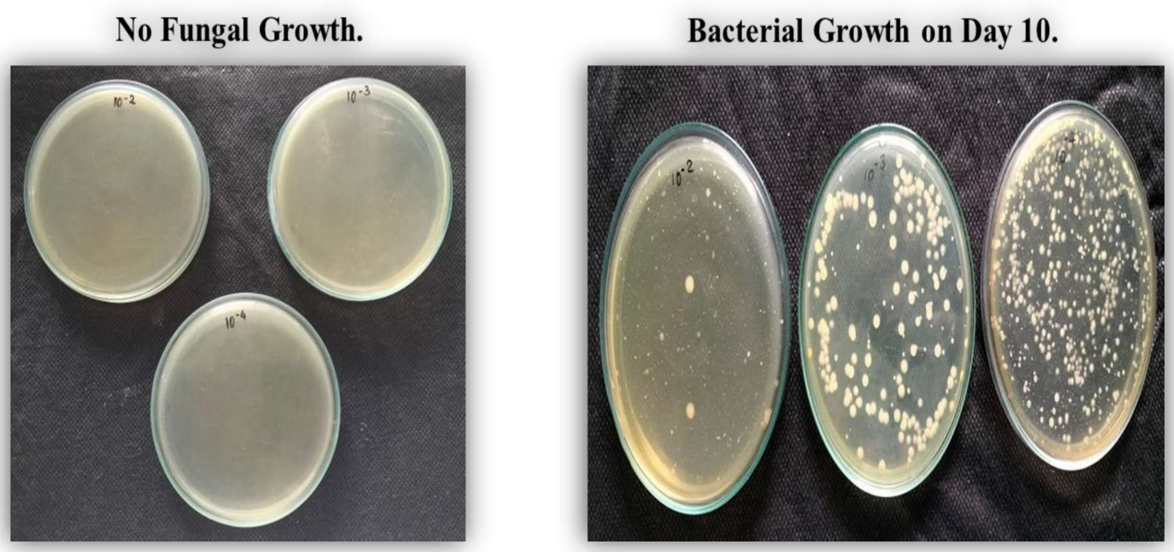


Plate. 4.5. Shelf-life Study of Probiotic Beverage Containing *U. lactuca* at 4±2℃

PHASE V

4.5 *In vitro* Bioavailability of Iron from the Developed Beverage using the Caco-2 Cell Model.

4.5.1 Cytotoxicity Study on the Caco-2 cell Model.

The cytotoxicity of the developed probiotic beverage Variant 1 was evaluated by using MTT assay on Caco-2 cell lines. The extent of cytotoxicity is represented by percent cell viability. Graph 4.13. depicts the percent cell viability of developed probiotic beverage sample V_1. Table. 4.29. depicts the percentage cell viability and IC_{50} value in the Caco-2 cell model.

Graph 4.13. Dose-response curve of V_1.

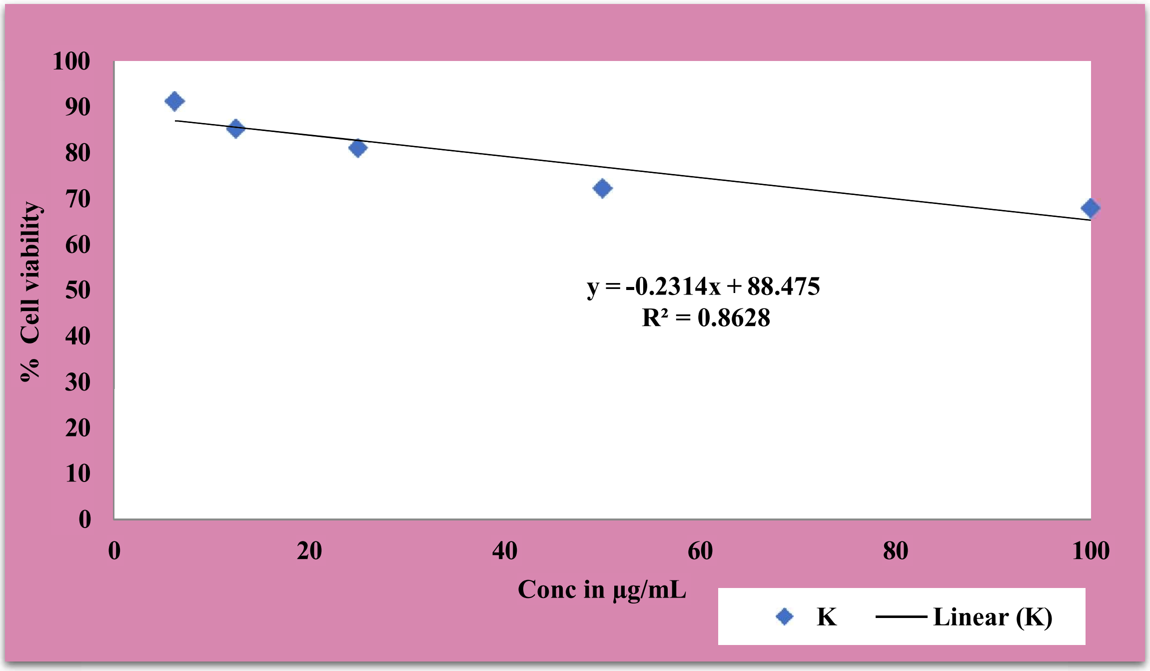


Table. 4.29. Cytotoxicity study on the Caco-2 cell model after 24 h incubation period.

Condition	% cell viability
Untreated	100.00
Std control	87.55
V_1-6.25µg	95.25
V_1-12.5µg	90.20
V_1-25µg	87.00
V_1-50µg	84.20
V_1-100µg	80.89
IC_{50}	35.96 µg/mL

It was observed that the cell lines had 84.2 % viability when supplemented with control samples of the beverage. The decrease in cell viability indicates that the cell lines did not proliferate after supplementation with the beverage sample. The decrease in proliferation of cell lines was due to the protective action of Ascorbic acid present in the beverage samples. The IC_{50} value is determined to be 35.96 µg/mL. This is the concentration of the substance at which cell viability is reduced by 50% compared to untreated control cells. Similar results were reported by Bedoya-Ramírez *et al.,* (2017) for Colombian fermented coffee beverage. The polymerization of phytochemicals could be the reason for the antiproliferative activity of the developed probiotic beverage against cancer cell lines. It was also reported that the flavonoids and their derivatives can cause cell cycle arrest, induce apoptosis and inhibit the cellular growth of cancer cells (Li *et al.,* 2018). The direct microscopic observation images of V_1 after 24 hours of incubation were depicted in the Plate. 4.6. Graph 4.14. shows the Cytotoxicity study on the Caco-2 cell model after 24 h incubation period.

Graph 4.14. Cytotoxicity study on the Caco-2 cell model after 24 h incubation period.

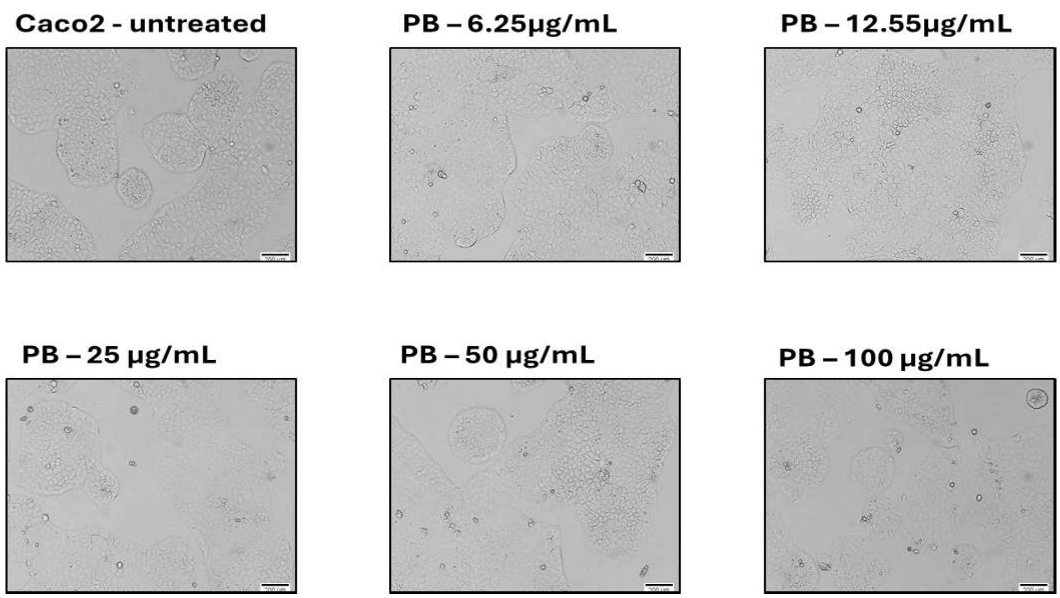


Plate.4.6. The effect of beverage dosage on the viability of Caco-2 cell lines (magnification 100x).

4.5.2. Iron Uptake Study in Caco-2 cell Model.

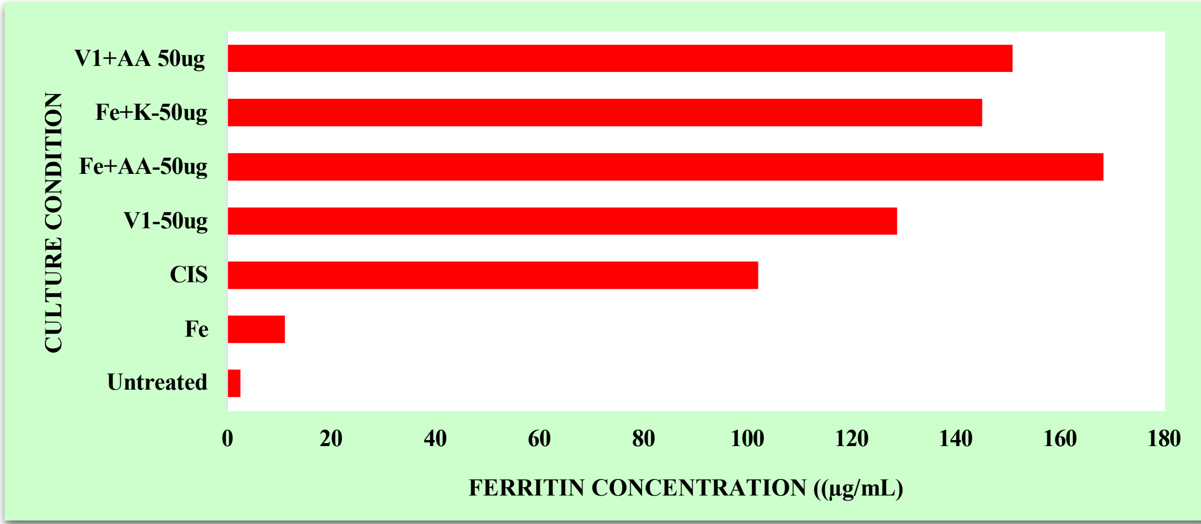
Table and Overlaid bar graph showed the Iron bioavailability in different culture conditions of the Caco-2 model. Each assay was performed in duplicate. In the current study, a low content of Ferritin was observed in individual groups, and it was enhanced with the combination of Fe. Iron in combination with 50 µg of Ascorbic acid showed 168.26±1.42 µg/mL of ferritin uptake, followed by 150.88±5.73 µg/mL of ferritin uptake by 50 µg of Variant 1 in combination with ascorbic acid. 50 µg of Iron and V_1 showed 145±3.45 µg/mL of ferritin uptake and 50 µg of Variant 1 showed 128.62±0.70 µg/mL. Iron and untreated cells showed 11.06±0.67 µg/mL and 2.52±0.47 µg/mL respectively. Effective Iron uptake was scored in Ascorbic acid and Sample V_1 with 50ug/mL concentration in Caco-2 cells and confirmed that Sample V_1 have the potential of Iron bioavailability in the Human intestinal model.

The most effective and peer-accepted *in vitro* iron bioavailability models are those that combine simulated gastrointestinal digestion with the culture of human intestinal epithelial cells or hepatocytes. An increase in ferritin is evidence of iron uptake by cells because cells produce ferritin in response to intracellular iron. In the current study, low content of Ferritin was observed in individual groups and it was enhanced with the combination of Fe. Effective Iron uptake was scored in Ascorbic acid and Sample V_1 with 50ug/mL concentration in Caco-2 cells and confirmed that Sample V_1 have the potential of Iron bioavailability in the Human intestinal model. The increased iron bioavailability in Sample V_1 may be due to the presence of Organic acids, Sugars and polyphenols which have a major role in the modulation of iron bioavailability. Table.4.30. and Graph 4.15. shows the cellular ferritin concentration observed in different culture conditions of Caco-2 cells.

Table.4.30. Cellular ferritin concentration observed in different culture conditions of Caco-2cells.

Culture condition	Ferritin conc (µg/mL)±SD	% increase of Bioavailability of Iron
Untreated	2.52±0.47	22.78
Fe	11.06±0.67	100
V_1-50µg	128.62±0.70	1162
Fe+AA-50µg	168.26±1.42	1521
V_1+AA 50µg	150.88±5.73	1364

Graph 4.15. Cellular ferritin concentration observed indifferent culture conditions of Caco-2cells.



Cellular Protein concentration

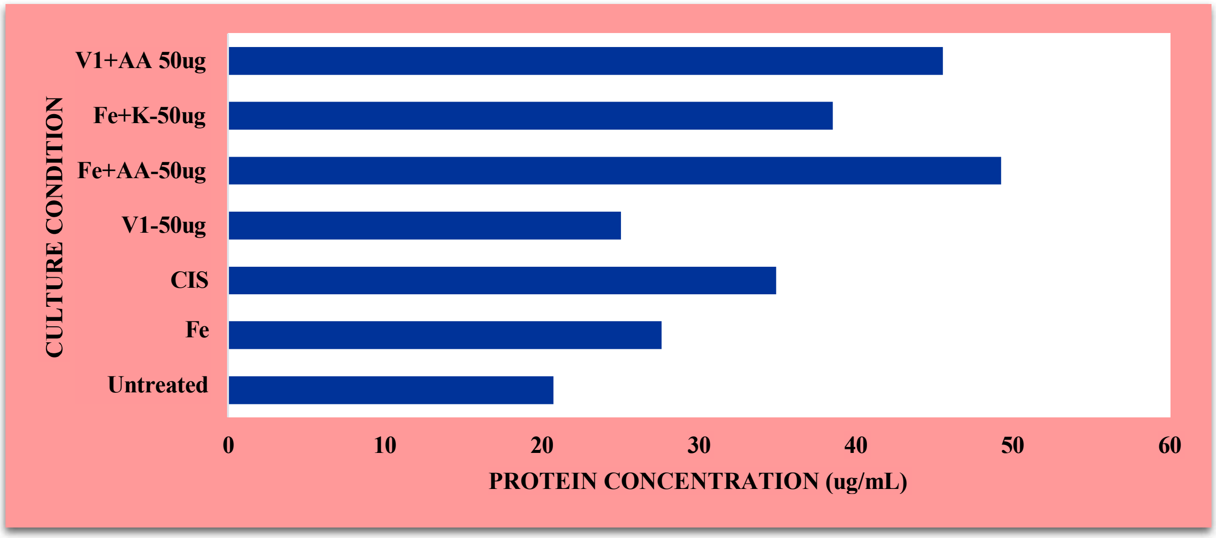
50µg of Iron with Ascorbic Acid has shown a protein concentration of 49.24±1.72 µg/mL, followed by V_1 in combination with ascorbic acid, which showed a protein concentration of 45.52±0.19 µg/mL. 50µg of Iron in combination with Variant 1 of the beverage demonstrated a protein concentration of 38.52±0.15 µg/mL, followed by Standard iron solution, Variant 1 and untreated samples which demonstrated a protein concentration value of 27.60±1.14 µg/mL, 25.02±0.88 µg/mL and 20.71±1.51 µg/mL respectively. Table. 4.31. shows the cellular protein concentration observed in different culture conditions of Caco-2 cells.

Graph 4.15. shows overlaid bar graph showed the Protein concentration in different culture conditions of Caco-2 model. Each assay was performed in duplicate and the experiments were repeated 1 time.

Table. 4.31. Cellular Protein concentration observed in different culture conditions of Caco-2 cells.

Culture condition	Protein conc (ug/mL)±SD
Untreated	20.71±1.51
Fe	27.60±1.14
V_1-50 µg	25.02±0.88
Fe+AA-50 µg	49.24±1.72
Fe+V_1-50 µg	38.52±0.15
V_1+AA 50 µg	45.52±0.19

Graph 4.15. Cellular Protein concentration observed in different culture conditions of Caco-2 cells.



Percentage increase of bioavailability of Iron in *in vitro* Caco-2 cell model.

Since Ferritin is a protein that stores iron within cells, measuring of ferritin concentration in Caco-2 cells after exposure to food sample how much iron has been absorbed by the cells. Higher ferritin levels indicate greater iron uptake. The iron uptake study in Caco 2 cell model recorded that iron bioavailability has increased more than 10 times in 50 ug of Variant 1 of the beverage. In the presence of 50ug of Ascorbic acid, 15 times increase of percentage iron bioavailability was observed and, when V_1 in the presence of Ascorbic acid showed a 13-fold increase in percent bioavailability of iron. Table.4.32. depicts the percent increase of bioavailable iron from the Caco-2 cell model.

Table. 4.32. Percent increase of bioavailable iron from the Caco-2 cell model.

S.No.	Culture condition	Ferritin conc (µg/mL)±SD	% Increase of Bioavailability of Iron
1	**Untreated**	2.52±0.47	22.78
2	**Fe**	11.06±0.67	100
3	**V1 - 50ug**	128.62±0.70	1162
4	**Iron+Ascorbic acid (AA)-50ug**	168.26±1.42	1521
5	**V_1+AA -50ug**	150.88±5.73	1364

From the foregoing results, it is evident that it was feasible to develop a probiotic beverage incorporating *Ulva Lactuca*. The beverage was nutrient-rich and the probiotic strain *L.reuteri* OP389067 demonstrated probiotic potentials in terms of tolerance towards pH, simulated gastric juice, bile juice and pancreatic juice. Also, the isolate demonstrated antibiotic susceptibility and activity against common food-borne bacteria. The nutrient and nutraceutical potentials of the developed probiotic beverage showed prominent antioxidant properties. The beverage was also rich in bioactive compounds, catering to the therapeutic attributes of the beverage. The *in vitro* bioavailability study of Iron using the Caco-2 cells demonstrated that the beverage showed good ferritin uptake in the presence of Ascorbic acid, and hence showed to be a good source of bioavailable iron.